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POLYNUCLEOTIDES ENCODING A NOVEL TESTIS-SPECIFIC TUBULIN  
TYROSINE-LIGASE-LIKE PROTEIN, BGS42

This application is a continuation-in-part application of non-provisional  
5 application U.S. Serial No. 10/615,659, filed July 9, 2003, which claims benefit to  
provisional application U.S. Serial No. 60/394,725 filed July 9, 2002, under 35 U.S.C.  
119(e).

FIELD OF THE INVENTION

10 The present invention provides novel polynucleotides encoding BGS-42  
polypeptides, fragments and homologues thereof. Also provided are vectors, host  
cells, antibodies, and recombinant and synthetic methods for producing said  
polypeptides. The invention further relates to diagnostic and therapeutic methods for  
applying these novel BGS-42 polypeptides to the diagnosis, treatment, and/or  
15 prevention of various diseases and/or disorders related to these polypeptides. The  
invention further relates to screening methods for identifying agonists and antagonists  
of the polynucleotides and polypeptides of the present invention.

BACKGROUND OF THE INVENTION

20 Tubulin, a subunit of microtubules, is subjected to specific enzymatic post-  
translational modifications, including cyclic tyrosine removal and addition at the  
COOH terminus of the alpha-subunit, during normal cellular metabolism. Tubulin is  
normally tyrosinated in cycling cells. It has previously been shown that de-tyrosinated  
tubulin accumulates in cancer cells during tumor progression in nude mice. Tubulin  
25 de-tyrosination, results from the suppression of tubulin tyrosine ligase and leads to an  
unbalanced activity of another enzyme, tubulin-carboxypeptidase. Apparently the loss  
of tyrosinate tubulin represents a strong selective advantage for cancer cells.

The occurrence and significance of tubulin de-tyrosination in human breast  
tumors has been investigated. Cancer cells with de-tyrosinated tubulin were observed  
30 in 53% of the tumors and were predominant in 19.4% of the tumors tested. Tubulin  
de-tyrosination has also been shown to correlate to a high degree of significance with  
markers of tumor aggressiveness (Mialhe et al., 2001).



There appears to be three genes described in the literature whose gene products could potentially possess tubulin tyrosine ligase activity, HOLLT (Genbank Accession No. gil6683745), TTLH\_Human (Tubulin tyrosine ligase-like protein; Genbank Accession No. gil20455371) and TTLL\_human (Tubulin tyrosine ligase-like protein 1; Genbank Accession No. gil20455347; Gene 257 1: 109-117 (2000); Genome Res. 11 (3), 422-435 (2001); and Nature 402 (6761), 489-495 (1999)). The regulation of these genes in normal cells, in addition to transformed cells remains to be determined as does the mechanism of their loss in enzymatic tyrosine-ligase activity during tumor progression. It has been proposed that a reversible phosphorylation event of TTL mediated by a protein kinase may be the basis of TTL loss of function (Idriss, 2001).

Using the above examples, it is clear the availability of a novel cloned tubulin tyrosine ligase protein provides an opportunity for further advancements in the physiological function of tubulin tyrosine ligase proteins, and may be useful for the identification of tubulin tyrosine ligase agonists, or stimulators (which might stimulate and/or bias tubulin tyrosine ligase action), as well as, in the identification of tubulin tyrosine ligase inhibitors. All of which might be therapeutically useful under different circumstances.

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells, in addition to their use in the production of BGS-42 polypeptides or peptides using recombinant techniques. Synthetic methods for producing the polypeptides and polynucleotides of the present invention are provided. Also provided are diagnostic methods for detecting diseases, disorders, and/or conditions related to the BGS-42 polypeptides and polynucleotides, and therapeutic methods for treating such diseases, disorders, and/or conditions. The invention further relates to screening methods for identifying binding partners of the polypeptides.

## BRIEF SUMMARY OF THE INVENTION

The present invention provides isolated nucleic acid molecules, that comprise, or alternatively consist of, a polynucleotide encoding the BGS-42 protein having the

amino acid sequence shown in Figures 1A-C (SEQ ID NO:2) or the amino acid sequence encoded by the contiguous sequence formed by the cDNA clones, BGS-42 clone A, B and C, deposited as ATCC Deposit Number PTA-4454 on June 12, 2002.

5 The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells, in addition to their use in the production of BGS-42 polypeptides or peptides using recombinant techniques. Synthetic methods for producing the polypeptides and polynucleotides of the present invention are provided. Also provided are diagnostic  
10 methods for detecting diseases, disorders, and/or conditions related to the BGS-42 polypeptides and polynucleotides, and therapeutic methods for treating such diseases, disorders, and/or conditions. The invention further relates to screening methods for identifying binding partners of the polypeptides.

The invention further provides an isolated BGS-42 polypeptide having an  
15 amino acid sequence encoded by a polynucleotide described herein.

The invention further relates to a polynucleotide encoding a polypeptide fragment of SEQ ID NO:2, or a polypeptide fragment encoded by the cDNA sequence included in the deposited clone, which is hybridizable to SEQ ID NO:1.

The invention further relates to a polynucleotide encoding a polypeptide  
20 domain of SEQ ID NO:2 or a polypeptide domain encoded by the cDNA sequence included in the deposited clone, which is hybridizable to SEQ ID NO:1.

The invention further relates to a polynucleotide encoding a polypeptide epitope of SEQ ID NO:2 or a polypeptide epitope encoded by the cDNA sequence included in the deposited clone, which is hybridizable to SEQ ID NO:1.

25 The invention further relates to a polynucleotide encoding a polypeptide of SEQ ID NO:2 or the cDNA sequence included in the deposited clone, which is hybridizable to SEQ ID NO:1, having biological activity.

The invention further relates to a polynucleotide which is a variant of SEQ ID NO:1.

30 The invention further relates to a polynucleotide which is an allelic variant of SEQ ID NO:1.



The invention further relates to a polynucleotide which encodes a species homologue of the SEQ ID NO:2.

The invention further relates to a polynucleotide which represents the complimentary sequence (antisense) of SEQ ID NO:1.

5       The invention further relates to a polynucleotide capable of hybridizing under stringent conditions to any one of the polynucleotides specified herein, wherein said polynucleotide does not hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence of only A residues or of only T residues.

10       The invention further relates to an isolated nucleic acid molecule of SEQ ID NO:2, wherein the polynucleotide fragment comprises a nucleotide sequence encoding an BGS-42 protein.

15       The invention further relates to an isolated nucleic acid molecule of SEQ ID NO:1, wherein the polynucleotide fragment comprises a nucleotide sequence encoding the sequence identified as SEQ ID NO:2 or the polypeptide encoded by the cDNA sequence included in the deposited clone, which is hybridizable to SEQ ID NO:1.

20       The invention further relates to an isolated nucleic acid molecule of SEQ ID NO:1, wherein the polynucleotide fragment comprises the entire nucleotide sequence of SEQ ID NO:1 or the cDNA sequence included in the deposited clone, which is hybridizable to SEQ ID NO:1.

      The invention further relates to an isolated nucleic acid molecule of SEQ ID NO:1, wherein the nucleotide sequence comprises sequential nucleotide deletions from either the C-terminus or the N-terminus.

25       The invention further relates to an isolated polypeptide comprising an amino acid sequence that comprises a polypeptide fragment of SEQ ID NO:2 or the encoded sequence included in the deposited clone.

      The invention further relates to a polypeptide fragment of SEQ ID NO:2 or the encoded sequence included in the deposited clone, having biological activity.

30       The invention further relates to a polypeptide domain of SEQ ID NO:2 or the encoded sequence included in the deposited clone.

      The invention further relates to a polypeptide epitope of SEQ ID NO:2 or the encoded sequence included in the deposited clone.

The invention further relates to a full length protein of SEQ ID NO:2 or the encoded sequence included in the deposited clone.

The invention further relates to a variant of SEQ ID NO:2.

The invention further relates to an allelic variant of SEQ ID NO:2. The  
5 invention further relates to a species homologue of SEQ ID NO:2.

The invention further relates to the isolated polypeptide of SEQ ID NO:2, wherein the full length protein comprises sequential amino acid deletions from either the C-terminus or the N-terminus.

The invention further relates to an isolated antibody that binds specifically to  
10 the isolated polypeptide of SEQ ID NO:2.

The invention further relates to a method for preventing, treating, or ameliorating a medical condition, comprising administering to a mammalian subject a therapeutically effective amount of the polypeptide of SEQ ID NO:2 or the polynucleotide of SEQ ID NO:1.

15 The invention further relates to a method of diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of (a) determining the presence or absence of a mutation in the polynucleotide of SEQ ID NO:1; and (b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or absence of said mutation.

20 The invention further relates to a method of diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of (a) determining the presence or amount of expression of the polypeptide of SEQ ID NO:2 in a biological sample; and (b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or amount of  
25 expression of the polypeptide.

The invention further relates to a method for identifying a binding partner to the polypeptide of SEQ ID NO:2 comprising the steps of (a) contacting the polypeptide of SEQ ID NO:2 with a binding partner; and (b) determining whether the binding partner effects an activity of the polypeptide.

30 The invention further relates to a gene corresponding to the cDNA sequence of SEQ ID NO:1.

The invention further relates to a method of identifying an activity in a biological assay, wherein the method comprises the steps of (a) expressing SEQ ID NO:1 in a cell, (b) isolating the supernatant; (c) detecting an activity in a biological assay; and (d) identifying the protein in the supernatant having the activity.

5       The invention further relates to a process for making polynucleotide sequences encoding gene products having altered SEQ ID NO:2 activity comprising the steps of (a) shuffling a nucleotide sequence of SEQ ID NO:1, (b) expressing the resulting shuffled nucleotide sequences and, (c) selecting for altered activity as compared to the activity of the gene product of said unmodified nucleotide sequence.

10       The invention further relates to a shuffled polynucleotide sequence produced by a shuffling process, wherein said shuffled DNA molecule encodes a gene product having enhanced tolerance to an inhibitor of SEQ ID NO:2 activity.

      The invention further relates to a method for preventing, treating, or ameliorating a medical condition with the polypeptide provided as SEQ ID NO:2, in  
15   addition to, its encoding nucleic acid, wherein the medical condition is a reproductive disorder

      The invention further relates to a method for preventing, treating, or ameliorating a medical condition with the polypeptide provided as SEQ ID NO:2, in addition to, its encoding nucleic acid, wherein the medical condition is a male  
20   reproductive disorder.

      The invention further relates to a method for preventing, treating, or ameliorating a medical condition with the polypeptide provided as SEQ ID NO:2, in addition to, its encoding nucleic acid, wherein the medical condition is a disorder related to aberrant tubulin regulation.

25       The invention further relates to a method for preventing, treating, or ameliorating a medical condition with the polypeptide provided as SEQ ID NO:2, in addition to, its encoding nucleic acid, wherein the medical condition is a disorder related to aberrant tubulin tyrosinization.

      The invention further relates to a method for preventing, treating, or  
30   ameliorating a medical condition with the polypeptide provided as SEQ ID NO:2, in addition to, its encoding nucleic acid, wherein the medical condition is a proliferative disorder.

The invention further relates to a method for preventing, treating, or ameliorating a medical condition with the polypeptide provided as SEQ ID NO:2, in addition to, its encoding nucleic acid, wherein the medical condition is a proliferative disorder of the testis.

5       The present invention also relates to an isolated polynucleotide consisting of a portion of the human BGS-42 gene consisting of at least 8 bases, specifically excluding Genbank Accession Nos. BM808637; BM808516; AA144756; BE102364; and/or AI026623.

10       The present invention also relates to an isolated polynucleotide consisting of a nucleotide sequence encoding a fragment of the human BGS-42 protein, wherein said fragment displays one or more functional activities specifically excluding Genbank Accession Nos. BM808637; BM808516; AA144756; BE102364; and/or AI026623.

15       The present invention also relates to the polynucleotide of SEQ ID NO:1 consisting of at least 10 to 50 bases, wherein said at least 10 to 50 bases specifically exclude the polynucleotide sequence of Genbank Accession Nos. BM808637; BM808516; AA144756; BE102364; and/or AI026623.

20       The present invention also relates to the polynucleotide of SEQ ID NO:1 consisting of at least 15 to 100 bases, wherein said at least 15 to 100 bases specifically exclude the polynucleotide sequence of Genbank Accession Nos. BM808637; BM808516; AA144756; BE102364; and/or AI026623.

25       The present invention also relates to the polynucleotide of SEQ ID NO:1 consisting of at least 100 to 1000 bases, wherein said at least 100 to 1000 bases specifically exclude the polynucleotide sequence of Genbank Accession Nos. BM808637; BM808516; AA144756; BE102364; and/or AI026623.

30       The present invention also relates to an isolated polypeptide fragment of the human BGS-42 protein, wherein said polypeptide fragment does not consist of the polypeptide encoded by the polynucleotide sequence of Genbank Accession Nos. BM808637; BM808516; AA144756; BE102364; and/or AI026623.

30       The invention further relates to a method of identifying a compound that modulates the biological activity of BGS-42, comprising the steps of, (a) combining a candidate modulator compound with BGS-42 having the sequence set forth in one or

more of SEQ ID NO:2; and (b) measuring an effect of the candidate modulator compound on the activity of BGS-42.

5 The invention further relates to a method of identifying a compound that modulates the biological activity of a tubulin tyrosine ligase protein, comprising the steps of, (a) combining a candidate modulator compound with a host cell expressing BGS-42 having the sequence as set forth in SEQ ID NO:2; and, (b) measuring an effect of the candidate modulator compound on the activity of the expressed BGS-42.

10 The invention further relates to a method of identifying a compound that modulates the biological activity of BGS-42, comprising the steps of, (a) combining a candidate modulator compound with a host cell containing a vector described herein, wherein BGS-42 is expressed by the cell; and, (b) measuring an effect of the candidate modulator compound on the activity of the expressed BGS-42.

The invention further relates to a method of screening for a compound that is capable of modulating the biological activity of BGS-42, comprising the steps of: (a) providing a host cell described herein; (b) determining the biological activity of BGS-42 in the absence of a modulator compound; (c) contacting the cell with the modulator compound; and (d) determining the biological activity of BGS-42 in the presence of the modulator compound; wherein a difference between the activity of BGS-42 in the presence of the modulator compound and in the absence of the modulator compound indicates a modulating effect of the compound.

20 The invention further relates to a compound that modulates the biological activity of human BGS-42 as identified by the methods described herein.

The invention further relates to a polynucleotide encoding a polypeptide from about amino acids 73 to about amino acid 365 of SEQ ID NO:2, wherein said polynucleotide encodes a tubulin tyrosine ligase domain of BGS-42.

25 The invention further relates to a polypeptide from about amino acids 73 to about amino acid 365 of SEQ ID NO:2, wherein said amino acids correspond to a tubulin tyrosine ligase domain of BGS-42.

The invention further relates to the isolated polypeptide of SEQ ID NO:2, wherein said polypeptide is phosphorylated.

The invention further relates to the isolated polypeptide of SEQ ID NO:2, wherein said polypeptide is phosphorylated at the cAMP-dependent protein kinase phosphorylation located at amino acid 306 to amino acid 309 of SEQ ID NO:2.

5 The invention further relates to an isolated polynucleotide of SEQ ID NO:27, wherein said polynucleotide represents at least a portion of the endogenous promoter of nucleotides 153 to 1775 of SEQ ID NO:1.

The invention further relates to a vector comprising at least a portion of the isolated polynucleotide of SEQ ID NO:27, wherein said polynucleotide represents at least a portion of the endogenous promoter of nucleotides 153 to 1775 of SEQ ID  
10 NO:1.

The invention further relates to a vector comprising at least a portion of the isolated polynucleotide of SEQ ID NO:27 in operable linkage to the encoding sequence of at least a portion of a gene, wherein said polynucleotide represents at least a portion of the endogenous promoter of nucleotides 153 to 1775 of SEQ ID  
15 NO:1.

The invention further relates to a method of controlling the expression of a polypeptide comprising the steps of: a) inserting the encoding polynucleotide sequence of said polypeptide into a vector comprising at least a portion of the isolated polynucleotide of SEQ ID NO:27, wherein said encoding polynucleotide sequence is  
20 downstream of the isolated polynucleotide of SEQ ID NO:27 such that the expression of said polypeptide is controlled by the isolated polynucleotide of SEQ ID NO:27; and b) inserting said vector into a host cell under conditions in which the polypeptide will be expressed under the control of the isolated polynucleotide of SEQ ID NO:27.

The invention further relates to an isolated polynucleotide of SEQ ID NO:27,  
25 wherein said polynucleotide represents at least a portion of the endogenous promoter of nucleotides 153 to 1775 of SEQ ID NO:1, and wherein said polynucleotide is mutated such that at least one or more of the CpG islands contained therein are not capable of being methylated.

The invention further relates to an isolated polynucleotide of SEQ ID NO:27,  
30 wherein said polynucleotide represents at least a portion of the endogenous promoter of nucleotides 153 to 1775 of SEQ ID NO:1, and wherein said polynucleotide is

mutated such that the CpG islands contained therein have an increased degree of methylation.

5 The invention further relates to a vector comprising at least a portion of the isolated polynucleotide of SEQ ID NO:27, wherein said polynucleotide represents at least a portion of the endogenous promoter of nucleotides 153 to 1775 of SEQ ID NO:1, and wherein said polynucleotide is mutated such that at least one or more of the CpG islands contained therein are not capable of being methylated.

10 The invention further relates to a vector comprising at least a portion of the isolated polynucleotide of SEQ ID NO:27, wherein said polynucleotide represents at least a portion of the endogenous promoter of nucleotides 153 to 1775 of SEQ ID NO:1, and wherein said polynucleotide is mutated such that the CpG islands contained therein have an increased degree of methylation.

15 The invention further relates to a vector comprising at least a portion of the isolated polynucleotide of SEQ ID NO:27 in operable linkage to the encoding sequence of at least a portion of a gene, wherein said polynucleotide represents at least a portion of the endogenous promoter of nucleotides 153 to 1775 of SEQ ID NO:1, and wherein said polynucleotide is mutated such that at least one or more of the CpG islands contained therein are not capable of being methylated.

20 The invention further relates to a vector comprising at least a portion of the isolated polynucleotide of SEQ ID NO:27 in operable linkage to the encoding sequence of at least a portion of a gene, wherein said polynucleotide represents at least a portion of the endogenous promoter of nucleotides 153 to 1775 of SEQ ID NO:1, and wherein said polynucleotide is mutated such that the CpG islands contained therein have an increased degree of methylation.

25 The invention further relates to a method of controlling the expression of a polypeptide comprising the steps of: a) inserting the encoding polynucleotide sequence of said polypeptide into a vector comprising at least a portion of the isolated polynucleotide of SEQ ID NO:27 wherein said polynucleotide is mutated such that at least one or more of the CpG islands contained therein are not capable of being  
30 methylated, wherein said encoding polynucleotide sequence is downstream of the isolated polynucleotide of SEQ ID NO:27 such that the expression of said polypeptide is controlled by the isolated polynucleotide of SEQ ID NO:27; and b) inserting said

vector into a host cell under conditions in which the polypeptide will be expressed under the control of the isolated polynucleotide of SEQ ID NO:27.

5 The invention further relates to a method of controlling the expression of a polypeptide comprising the steps of: a) inserting the encoding polynucleotide sequence of said polypeptide into a vector comprising at least a portion of the isolated polynucleotide of SEQ ID NO:27 wherein said polynucleotide is mutated such that the CpG islands contained therein have an increased degree of methylation, wherein said encoding polynucleotide sequence is downstream of the isolated polynucleotide of SEQ ID NO:27 such that the expression of said polypeptide is controlled by the  
10 isolated polynucleotide of SEQ ID NO:27; and b) inserting said vector into a host cell under conditions in which the polypeptide will be expressed under the control of the isolated polynucleotide of SEQ ID NO:27.

The invention further relates to a method of ameliorating, preventing, and/or decreasing the level of BGS-42 expression inhibition observed in proliferating cells  
15 and tissues by altering the methylation capacity of the endogenous BGS-42 promoter sequence wherein said methylation capacity is decreased.

The invention further relates to a method of treating, ameliorating, and/or preventing a proliferative medical condition, such as testicular, lung, and colon cancer, comprising the step of increasing the expression level of the BGS-42  
20 polypeptide within the proliferative tissues.

The invention further relates to a method of transforming a proliferative tissue and/or cell to a more normal phenotype in vitro, comprising the step of increasing the expression level of the BGS-42 polypeptide within the proliferative tissues.

25 The invention relates to a method of diagnosing a proliferative condition comprising the steps of a) detecting the expression of the BGS-42 polypeptide using primers directed to the encoding polynucleotide; and b) comparing the BGS-42 expression level to the expression of a control gene; wherein a low level of BGS-42 expression corresponds to the detection of a proliferative tissue or cell, and wherein a high level of expression corresponds to the detection of a normal tissue or cell.

30 The invention further relates to an isolated polynucleotide representing at least a portion of the BGS-42 gene, comprising at least a portion of the polynucleotide



provided as SEQ ID NO:27 in operable linkage to nucleotides 153 to 1775 of SEQ ID NO:1.

5 The present invention also provides structure coordinates of the homology model of the BGS-42 polypeptide (SEQ ID NO:2) provided in Figure 13. The complete coordinates are listed in Table IV. The model of the present invention further provide a basis for designing stimulators and inhibitors or antagonists of one or more of the biological functions of BGS-42, or of mutants with altered ligand binding specificity.

10 The invention also provides a machine readable storage medium which comprises the structure coordinates of BGS-42, including all or any parts conserved ligase regions. Such storage medium encoded with these data are capable of displaying on a computer screen or similar viewing device, a three-dimensional graphical representation of a molecule or molecular complex which comprises said regions or similarly shaped homologous regions.

15 The invention also provides a machine-readable data storage medium, comprising a data storage material encoded with machine readable data, wherein the data is defined by the structure coordinates of the model BGS-42 according to Table IV or a homologue of said model, wherein said homologue comprises any kind of surrogate atoms that have a root mean square deviation from the backbone atoms of  
20 the complex of not more than about 4.0, 3.0, 2.0, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 Angstroms.

The invention also provides a model comprising all or any part of the model defined by structure coordinates of BGS-42 according to Table IV, or a mutant or homologue of said molecule or molecular complex.

25 The invention also provides a method for identifying a mutant of BGS-42 with altered biological properties, function, or reactivity, the method comprising one or more of the following steps: (a) use of the model or a homologue of said model according to Table IV, for the design of protein mutants with altered biological function or properties which exhibit any combination of therapeutic effects described  
30 herein; and/or (b) use of the model or a homologue of said model, for the design of a protein with mutations in the ligand binding/active site region comprised of the amino acids K143, G150, Q181, Y183, D196, D316, E281, and/or N283 of SEQ ID NO:2

according to Table IV with altered biological function or properties which exhibit any combination of therapeutic effects described herein.

5 The method also relates to a method for identifying modulators of BGS-42 biological properties, function, or reactivity, the method comprising the step of modeling test compounds that fit spatially into the active site region defined by all or any portion of residues K143, G150, Q181, Y183, D196, D316, E281, and N283 of the three-dimensional structural model according to Table IV, or using a homologue or portion thereof, or analogue in which the original C, N, and O atoms have been replaced with other elements

10 The invention also provides methods for designing, evaluating and identifying compounds which bind to all or parts of the aforementioned regions. The methods include three dimensional model building (homology modeling) and methods of computer assisted-drug design which can be used to identify compounds which bind or modulate the forementioned regions of the BGS-42 polypeptide. Such compounds  
15 are potential inhibitors of BGS-42 or its homologues.

The invention also relates to a method of using said structure coordinates as set forth in Table IV to identify structural and chemical features of BGS-42; employing identified structural or chemical features to design or select compounds as potential BGS-42 modulators; employing the three-dimensional structural model to  
20 design or select compounds as potential BGS-42 modulators; synthesizing the potential BGS-42 modulators; screening the potential BGS-42 modulators in an assay characterized by binding of a protein to the BGS-42. The invention also relates to said method wherein the potential BGS-42 modulator is selected from a database. The invention further relates to said method wherein the potential BGS-42 modulator is  
25 designed de novo. The invention further relates to a method wherein the potential BGS-42 modulator is designed from a known modulator of activity.

#### BRIEF DESCRIPTION OF THE FIGURES/DRAWINGS

30 Figures 1A-C show the polynucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of the novel human tubulin tyrosine ligase protein, BGS-42, of the present invention. The standard one-letter abbreviation for amino acids is used to illustrate the deduced amino acid sequence. The polynucleotide

sequence contains a sequence of 1838 nucleotides (SEQ ID NO:1), encoding a polypeptide of 541 amino acids (SEQ ID NO:2). An analysis of the BGS-42 polypeptide determined that it comprised the following features: a tubulin-tyrosine ligase family domain (TTL) located from about amino acid 73 to about amino acid 365 (TTL1; SEQ ID NO:14) of SEQ ID NO:2 (Figures 1A-C) represented by single underlining; conserved cysteine residues located at amino acid 155, 238, 347, 363, and/or 388 of SEQ ID NO:2 represented by shading; and a conserved cAMP-dependent protein kinase phosphorylation site located at amino acid 306 to amino acid 309 of SEQ ID NO:2 (Figures 1A-C) represented by double underlining.

Figure 2 shows the partial polynucleotide sequence (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) of the novel human tubulin tyrosine ligase protein, BGS-42, of the present invention. The standard one-letter abbreviation for amino acids is used to illustrate the deduced amino acid sequence. The polynucleotide sequence contains a sequence of 726 nucleotides (SEQ ID NO:3), encoding a polypeptide of 242 amino acids (SEQ ID NO:4).

Figures 3A-B show the regions of identity and similarity between the encoded BGS-42 protein (SEQ ID NO:2) to other tubulin tyrosine ligase proteins, specifically, the human HOTTL protein (HOTTL; Genbank Accession No:gil6683745; SEQ ID NO:5); the human tubulin tyrosine ligase-like protein (TTLH\_HUMAN; Genbank Accession No:gil20455371; SEQ ID NO:7); the human tubulin tyrosine ligase-like protein 1 (TTLL\_HUMAN; Genbank Accession No:gil20455347; SEQ ID NO:8); and the pig tubulin tyrosine ligase protein (TTL\_PIG; Genbank Accession No:gil423218; SEQ ID NO:6). The alignment was performed using the CLUSTALW algorithm using default parameters as described herein (Vector NTI suite of programs). The darkly shaded amino acids represent regions of matching identity. The lightly shaded amino acids represent regions of matching similarity. Dots (“•”) between residues indicate gapped regions of non-identity for the aligned polypeptides. The conserved cysteines between BGS-42 and the other tubulin tyrosine ligase proteins are noted and described herein.

Figure 4 shows the regions of local identity and similarity between the encoded BGS-42 protein (SEQ ID NO:2) to the Pfam TTL Tubulin-tyrosine ligase family consensus model sequence (TTL; Pfam Accession No: PF03133; SEQ ID

NO:26). The query ("Q") sequence represents the local matching sequence of the BGS-42 protein (SEQ ID NO:2), whereas the target ("T") represents the human TTL Tubulin-tyrosine ligase family consensus model sequence. The alignment was performed using the BLAST2 algorithm according to default parameters (SF Altschul, et al., Nucleic Acids Res 25:3389-3402, 1997). The amino acids between the query and target sequences represent matching identical amino acids between the two sequences. Plus signs ("+") between the query and target sequences represent similar amino acids between the two sequences. Dots ("•") between the query and target sequences indicate regions of non-identity for the aligned polypeptides. Capital letters of the target sequence in the alignment represent the most conserved residues of the TTL domain. The conserved cysteine between BGS-42 and the consensus TTL Tubulin-tyrosine ligase family polypeptide sequence is noted.

Figure 5A shows the polynucleotide sequence (SEQ ID NO:9) of clone A of BGS-42 that was isolated and used to arrive at the final consensus polynucleotide sequence of the full-length BGS-42, of the present invention. The polynucleotide sequence contains a sequence of 1939 nucleotides (SEQ ID NO:9).

Figure 5B shows the polynucleotide sequence (SEQ ID NO:10) of clone B of BGS-42 that was isolated and used to arrive at the final consensus polynucleotide sequence of the full-length BGS-42, of the present invention. The polynucleotide sequence contains a sequence of 1859 nucleotides (SEQ ID NO:10).

Figure 5C shows the polynucleotide sequence (SEQ ID NO:11) of clone C of BGS-42 that was isolated and used to arrive at the final consensus polynucleotide sequence of the full-length BGS-42, of the present invention. The polynucleotide sequence contains a sequence of 3465 nucleotides (SEQ ID NO:11).

Figure 6A-D show the consensus polynucleotide sequence (SEQ ID NO:12) and deduced amino acid sequence (SEQ ID NO:13) of the novel human tubulin tyrosine ligase protein, BGS-42, of the present invention based upon the contig formed by clones A, B, and C (SEQ ID NO:9, 10, and 11, respectively). The standard one-letter abbreviation for amino acids is used to illustrate the deduced amino acid sequence. The polynucleotide sequence contains a sequence of 3554 nucleotides (SEQ ID NO:12), encoding a polypeptide of 541 amino acids (SEQ ID NO:13).

Figures 7A-B shows the polynucleotide sequence (SEQ ID NO:27) of the upstream promoter sequence of the BGS-42 gene. The polynucleotide sequence contains a sequence of 2241 nucleotides with nucleotides –2057 to –1 representing the upstream promoter sequence. The location of the initiating start codon (nucleotide position +1 of SEQ ID NO:27)) and translation of a portion of the BGS-42 polynucleotide are shown. Analysis of the BGS-42 promoter region led to the identification of three CpG islands located from about nucleotide –1968 to about –1746; from about nucleotide –1232 to about –936; and/or from about nucleotide –727 to about –470 of SEQ ID NO:27 represented by double underlining. The location of the CpG islands was determined using the algorithm described by D.T and P.A.J., PNAS 99(6):3740-5 (2002).

Figure 8 shows an expression profile of the novel human tubulin tyrosine ligase protein, BGS-42, of the present invention. The figure illustrates the relative expression level of BGS-42 amongst various mRNA tissue sources. As shown, transcripts corresponding to BGS-42 expressed predominately in testis. The BGS-42 polypeptide was also expressed significantly in small intestine, stomach, spinal cord, and to a lesser extent, in brain, and liver. Expression data was obtained by measuring the steady state BGS-42 mRNA levels by quantitative PCR using the PCR primer pair provided as SEQ ID NO:28 and 29 as described in Example 5 herein.

Figure 9 shows an expression profile of the novel human tubulin tyrosine ligase protein, BGS-42, of the present invention. The figure illustrates the relative expression level of BGS-42 amongst various normal and tumor mRNA tissue sources. As shown, transcripts corresponding to BGS-42 showed differential expression predominately in lung compared to tumor lung tissue, with a 25 fold decrease in expression observed in lung tumors relative to normal lung tissue. This apparent loss of BGS-42 expression in tumor tissues relative to normal tissues suggests BGS-42 may play a role in tumor suppression, either directly or indirectly. Expression data was obtained by measuring the steady state BGS-42 mRNA levels by quantitative PCR using the PCR primer pair provided as SEQ ID NO:28 and 29 as described in Example 5 herein.

Figure 10 shows an expanded expression profile of the human tubulin tyrosine ligase protein, BGS-42, of the present invention. The figure illustrates the relative

expression level of BGS-42 amongst various mRNA tissue sources. As shown, the BGS-42 polypeptide was expressed predominately in the vas deferens. Expression of BGS-42 was also significantly expressed in lymph gland, pituitary, placenta, and to a lesser extent, in other tissues as shown. Expression data was obtained by measuring the steady state BGS-42 mRNA levels by quantitative PCR using the PCR primer pair provided as SEQ ID NO:28 and 29, and Taqman probe (SEQ ID NO:30) as described in Example 6 herein.

Figure 11 shows an expanded expression profile of the human tubulin tyrosine ligase protein, BGS-42, of the present invention. The figure illustrates the relative expression level of BGS-42 amongst various both control and five tumor mRNA tissue sources. As shown, expression of the BGS-42 polypeptide was not observed in any of the five testis tumor samples. This apparent loss of BGS-42 expression in tumor tissues relative to normal tissues suggests BGS-42 may play a role in tumor suppression, either directly or indirectly. Expression data was obtained by measuring the steady state BGS-42 mRNA levels by quantitative PCR using the PCR primer pair provided as SEQ ID NO:28 and 29, and Taqman probe (SEQ ID NO:30) as described in Example 6 herein.

Figure 12 shows a table illustrating the percent identity and percent similarity between the BGS-42 polypeptide of the present invention with other tubulin tyrosine ligase proteins, specifically, the human HOTTL protein (HOTTL; Genbank Accession No:gil6683745; SEQ ID NO:5); the human tubulin tyrosine ligase-like protein (TTLH\_HUMAN; Genbank Accession No:gil20455371; SEQ ID NO:7); the human tubulin tyrosine ligase-like protein 1 (TTLL\_HUMAN; Genbank Accession No:gil20455347; SEQ ID NO:8); and the pig tubulin tyrosine ligase protein (TTL\_PIG; Genbank Accession No:gil423218; SEQ ID NO:6). The percent identity and percent similarity values were determined using the Gap algorithm using default parameters (Genetics Computer Group suite of programs; Needleman and Wunsch. J. Mol. Biol. 48; 443-453, 1970); GAP parameters: gap creation penalty: 8 and gap extension penalty: 2).

FIG. 13: Sequence alignment of the conceptual translated sequence of BGS-42 of the present invention (SEQ ID NO:2) with *Escherichia coli* glutathione synthetase, a member of the peptide synthases ADP-forming enzymes (acid-D-amino acid

ligases) (Protein Data Bank entry 1GSA; Genbank Accession No. gil 15803486; SEQ ID NO:103). The alignment was used as the basis for building the BGS-42 homology model described herein. The coordinates of the BGS-42 model are provided in Table IV. Amino acids defining the binding and active site regions in both the model and the  
5 1GSA structure are highlighted with an asterisk (“\*”). Amino acids defining key hydrophobic residues of the amino acid ligase structural motif as defined by Dideberg and Bertrand (Trends Biochem. Sci. 23:57-58, 1998) are highlighted with a caret (“^”).

FIG. 14 shows the three-dimensional homology model of amino acids M1 to  
10 R361 of the BGS-42 polypeptide of the present invention (SEQ ID NO:2). The model is based upon an alignment to a structural homologue *Escherichia coli* glutathione synthetase (Protein Data Bank entry 1GSA; Genbank Accession No. gil 15803486; SEQ ID NO:103) that was used as the basis for building the BGS-42 homology model. The coordinates of the BGS-42 model are provided in Table IV. The model  
15 does not include the structure of the loop region E216 to W262 of SEQ ID NO:2, nor the C-terminal region S362 to V501 of SEQ ID NO:2. Both regions were not able to be modeled using 1GSA as the template.

Table I provides a summary of the novel polypeptides and their encoding polynucleotides of the present invention.

20 Table II illustrates the preferred hybridization conditions for the polynucleotides of the present invention. Other hybridization conditions may be known in the art or are described elsewhere herein.

Table III provides a summary of various conservative substitutions encompassed by the present invention.

25 Table IV provides the structural coordinates of the three dimensional structure of the BGS-42 polypeptide of the present invention (SEQ ID NO:2).

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the  
30 following detailed description of the preferred embodiments of the invention and the Examples included herein.

The invention provides a novel human sequence that encodes a tubulin tyrosine ligase protein with substantial homology to other tubulin tyrosine ligase proteins. Members of the tubulin tyrosine ligase protein family serve as proliferation inhibitors whereby loss of tubulin tyrosine ligase activity results in cellular proliferation. Tubulin tyrosine ligases have been implicated in a number of diseases and/or disorders, which include, but are not limited to, cancer, aberrant cellular proliferation, and aberrant cellular differentiation. Moreover, members of the tubulin tyrosine ligase protein family have also been implicated in having anti-proliferative roles as described herein.

In the present invention, "isolated" refers to material removed from its original environment (e.g., the natural environment if it is naturally occurring), and thus is altered "by the hand of man" from its natural state. For example, an isolated polynucleotide could be part of a vector or a composition of matter, or could be contained within a cell, and still be "isolated" because that vector, composition of matter, or particular cell is not the original environment of the polynucleotide. The term "isolated" does not refer to genomic or cDNA libraries, whole cell total or mRNA preparations, genomic DNA preparations (including those separated by electrophoresis and transferred onto blots), sheared whole cell genomic DNA preparations or other compositions where the art demonstrates no distinguishing features of the polynucleotide/sequences of the present invention.

In specific embodiments, the polynucleotides of the invention are at least 15, at least 30, at least 50, at least 100, at least 125, at least 500, or at least 1000 continuous nucleotides but are less than or equal to 300 kb, 200 kb, 100 kb, 50 kb, 15 kb, 10 kb, 7.5 kb, 5 kb, 2.5 kb, 2.0 kb, or 1 kb, in length. In a further embodiment, polynucleotides of the invention comprise a portion of the coding sequences, as disclosed herein, but do not comprise all or a portion of any intron. In another embodiment, the polynucleotides comprising coding sequences do not contain coding sequences of a genomic flanking gene (i.e., 5' or 3' to the gene of interest in the genome). In other embodiments, the polynucleotides of the invention do not contain the coding sequence of more than 1000, 500, 250, 100, 50, 25, 20, 15, 10, 5, 4, 3, 2, or 1 genomic flanking gene(s).



In at least one embodiment, the polynucleotides comprise the 5' upstream non-coding promoter of the BGS-42 gene (SEQ ID NO:27).

As used herein, a "polynucleotide" refers to a molecule having a nucleic acid sequence contained in SEQ ID NO:1 or the cDNA contained within the clone deposited with the ATCC. For example, the polynucleotide can contain the nucleotide sequence of the full length cDNA sequence, including the 5' and 3' untranslated sequences, the coding region, with or without a signal sequence, the secreted protein coding region, as well as fragments, epitopes, domains, and variants of the nucleic acid sequence. Moreover, as used herein, a "polypeptide" refers to a molecule having the translated amino acid sequence generated from the polynucleotide as broadly defined.

In the present invention, the full length sequence identified as SEQ ID NO:1 was often generated by overlapping sequences contained in one or more clones (contig analysis). A representative clone containing all or most of the sequence for SEQ ID NO:1 was deposited with the American Type Culture Collection ("ATCC"). As shown in Table I, each clone is identified by a cDNA Clone ID (Identifier) and the ATCC Deposit Number. The ATCC is located at 10801 University Boulevard, Manassas, Virginia 20110-2209, USA. The ATCC deposit was made pursuant to the terms of the Budapest Treaty on the international recognition of the deposit of microorganisms for purposes of patent procedure. The deposited clone is inserted in the pSport1 (Life Technologies) using the NotI and SalI restriction endonuclease sites as described herein.

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373, preferably a Model 3700, from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA

molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide  
5 sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Using the information provided herein, such as the nucleotide sequence in  
10 Figures 1A-C (SEQ ID NO:1), a nucleic acid molecule of the present invention encoding the BGS-42 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the nucleic acid molecule described in Figures 1A-C (SEQ ID NO:1) was discovered in a mixture of human circular brain and testis  
15 first strand cDNA library.

A "polynucleotide" of the present invention also includes those polynucleotides capable of hybridizing, under stringent hybridization conditions, to sequences contained in SEQ ID NO:1, the complement thereof, or the cDNA within the clone deposited with the ATCC. "Stringent hybridization conditions" refers to an  
20 overnight incubation at 42 degree C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65 degree C.

Also contemplated are nucleic acid molecules that hybridize to the  
25 polynucleotides of the present invention at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, lower stringency conditions include an overnight  
30 incubation at 37 degree C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH<sub>2</sub>PO<sub>4</sub>; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 ug/ml salmon sperm blocking DNA; followed by washes at 50 degree C with 1XSSPE,

0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC).

Note that variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

Of course, a polynucleotide which hybridizes only to polyA<sup>+</sup> sequences (such as any 3' terminal polyA<sup>+</sup> tract of a cDNA shown in the sequence listing), or to a complementary stretch of T (or U) residues, would not be included in the definition of "polynucleotide" since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone generated using oligo dT as a primer).

The polynucleotide of the present invention can be composed of any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. For example, polynucleotides can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, the polynucleotide can be composed of triple-stranded regions comprising RNA or DNA or both RNA and DNA. A polynucleotide may also contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

The polypeptide of the present invention can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide

isosteres, and may contain amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, *Proteins - Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993); *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al., *Meth Enzymol* 182:626-646 (1990); Rattan et al., *Ann NY Acad Sci* 663:48-62 (1992).)

"SEQ ID NO:1" refers to a polynucleotide sequence while "SEQ ID NO:2" refers to a polypeptide sequence, both sequences are identified by an integer specified in Table I.

"A polypeptide having biological activity" refers to polypeptides exhibiting activity similar, but not necessarily identical to, an activity of a polypeptide of the

present invention, including mature forms, as measured in a particular biological assay, with or without dose dependency. In the case where dose dependency does exist, it need not be identical to that of the polypeptide, but rather substantially similar to the dose-dependence in a given activity as compared to the polypeptide of the present invention (i.e., the candidate polypeptide will exhibit greater activity or not  
5 more than about 25-fold less and, preferably, not more than about tenfold less activity, and most preferably, not more than about three-fold less activity relative to the polypeptide of the present invention.)

As will be appreciated by the skilled practitioner, should the amino acid  
10 fragment comprise an antigenic epitope, for example, biological function *per se* need not be maintained. The terms BGS-42 polypeptide and BGS-42 protein are used interchangeably herein to refer to the encoded product of the BGS-42 nucleic acid sequence according to the present invention.

As used herein the terms “modulate” or “modulates” refer to an increase or  
15 decrease in the amount, quality or effect of a particular activity, DNA, RNA, or protein. The definition of “modulate” or “modulates” as used herein is meant to encompass agonists and/or antagonists of a particular activity, DNA, RNA, or protein.

It is another aspect of the present invention to provide modulators of the BGS-42 protein and BGS-42 peptide targets which can affect the function or activity of  
20 BGS-42 in a cell in which BGS-42 function or activity is to be modulated or affected. In addition, modulators of BGS-42 can affect downstream systems and molecules that are regulated by, or which interact with, BGS-42 in the cell. Modulators of BGS-42 include compounds, materials, agents, drugs, and the like, that antagonize, inhibit, reduce, block, suppress, diminish, decrease, or eliminate BGS-42 function and/or  
25 activity. Such compounds, materials, agents, drugs and the like can be collectively termed “antagonists”. Alternatively, modulators of BGS-42 include compounds, materials, agents, drugs, and the like, that agonize, enhance, increase, augment, or amplify BGS-42 function in a cell. Such compounds, materials, agents, drugs and the like can be collectively termed “agonists”.

30 The term “organism” as referred to herein is meant to encompass any organism referenced herein, though preferably to eukaryotic organisms, more preferably to mammals, and most preferably to humans.

The present invention encompasses the identification of proteins, nucleic acids, or other molecules, that bind to polypeptides and polynucleotides of the present invention (for example, in a receptor-ligand interaction). The polynucleotides of the present invention can also be used in interaction trap assays (such as, for example, that described by Ozenberger and Young (Mol Endocrinol., 9(10):1321-9, (1995); and  
5 Ann. N. Y. Acad. Sci., 7;766:279-81, (1995)).

The polynucleotide and polypeptides of the present invention are useful as probes for the identification and isolation of full-length cDNAs and/or genomic DNA which correspond to the polynucleotides of the present invention, as probes to  
10 hybridize and discover novel, related DNA sequences, as probes for positional cloning of this or a related sequence, as probe to "subtract-out" known sequences in the process of discovering other novel polynucleotides, as probes to quantify gene expression, and as probes for microarrays.

In addition, polynucleotides and polypeptides of the present invention may  
15 comprise one, two, three, four, five, six, seven, eight, or more membrane domains.

Also, in preferred embodiments the present invention provides methods for further refining the biological function of the polynucleotides and/or polypeptides of the present invention.

Specifically, the invention provides methods for using the polynucleotides and  
20 polypeptides of the invention to identify orthologs, homologs, paralogs, variants, and/or allelic variants of the invention. Also provided are methods of using the polynucleotides and polypeptides of the invention to identify the entire coding region of the invention, non-coding regions of the invention, regulatory sequences of the invention, and secreted, mature, pro-, prepro-, forms of the invention (as applicable).

In preferred embodiments, the invention provides methods for identifying the  
25 glycosylation sites inherent in the polynucleotides and polypeptides of the invention, and the subsequent alteration, deletion, and/or addition of said sites for a number of desirable characteristics which include, but are not limited to, augmentation of protein folding, inhibition of protein aggregation, regulation of intracellular trafficking to  
30 organelles, increasing resistance to proteolysis, modulation of protein antigenicity, and mediation of intercellular adhesion.

In further preferred embodiments, methods are provided for evolving the polynucleotides and polypeptides of the present invention using molecular evolution techniques in an effort to create and identify novel variants with desired structural, functional, and/or physical characteristics.

5       The present invention further provides for other experimental methods and procedures currently available to derive functional assignments. These procedures include but are not limited to spotting of clones on arrays, micro-array technology, PCR based methods (e.g., quantitative PCR), anti-sense methodology, gene knockout experiments, and other procedures that could use sequence information from clones to  
10   build a primer or a hybrid partner.

#### Polynucleotides and Polypeptides of the Invention

##### Features of the Polypeptide Encoded by Gene No:1

The polypeptide of this gene provided as SEQ ID NO:2 (Figures 1A-C), encoded by the polynucleotide sequence according to SEQ ID NO:1 (Figures 1A-C),  
15   and/or encoded by the polynucleotide contained within the deposited clone, BGS-42, has significant homology at the nucleotide and amino acid level to a number of tubulin tyrosine ligase proteins, which include, for example, the human HOTTL protein (HOTTL; Genbank Accession No:gil6683745; SEQ ID NO:5); the human tubulin tyrosine ligase-like protein (TTLH\_HUMAN; Genbank Accession  
20   No:gil20455371; SEQ ID NO:7); the human tubulin tyrosine ligase-like protein 1 (TTLL\_HUMAN; Genbank Accession No:gil20455347; SEQ ID NO:8); and the pig tubulin tyrosine ligase protein (TTL\_PIG; Genbank Accession No:gil423218; SEQ ID NO:6). An alignment of the BGS-42 polypeptide with these proteins is provided in Figures 3A-B.

25       The determined nucleotide sequence of the BGS-42 cDNA in Figures 1A-C (SEQ ID NO:1) contains an open reading frame encoding a protein of about 541 amino acid residues, with a deduced molecular weight of about 59.9 kDa. The amino acid sequence of the predicted BGS-42 polypeptide is shown in Figures 1A-C (SEQ ID NO:2). The BGS-42 protein shown in Figures 1A-C was determined to share  
30   significant identity and similarity to several known tubulin tyrosine ligase proteins. Specifically, the BGS-42 protein shown in Figures 1A-C was determined to be about 57.5% identical and 65.5% similar to the human HOTTL protein (HOTTL; Genbank

Accession No:gil6683745; SEQ ID NO:5); to be about 53.3% identical and 61.4% similar to the human tubulin tyrosine ligase-like protein (TTLH\_HUMAN; Genbank Accession No:gil20455371; SEQ ID NO:7); to be about 27.4% identical and 38.4% similar to the human tubulin tyrosine ligase-like protein 1 (TTLL\_HUMAN; Genbank  
 5 Accession No:gil20455347; SEQ ID NO:8); and to be about 29.4% identical and 40.0% similar to the pig tubulin tyrosine ligase protein (TTL\_PIG; Genbank Accession No:gil423218; SEQ ID NO:6); as shown in Figure 7.

The human tubulin tyrosine ligase-like protein 1 (TTLL\_HUMAN; Genbank Accession No:gil20455347; SEQ ID NO:8) is a tubulin tyrosine ligase protein that has  
 10 been mapped to chromosome region 22q13.1 (Gene 257 (1), 109-117 (2000)).

The pig tubulin tyrosine ligase protein (TTL\_PIG; Genbank Accession No:gil423218; SEQ ID NO:6) is a validated tubulin tyrosine ligase protein that catalyzes the ATP-dependent posttranslational addition of a tyrosine to the carboxyterminal end of detyrosinated alpha-tubulin (J. Cell Biol. 120 (3), 725-732  
 15 (1993)).

The BGS-42 polypeptide was predicted to comprise one TTL family domain (TTL1) located from about amino acid 73 to about amino acid 365 (TTL1; SEQ ID NO:14) of SEQ ID NO:2 (Figures 1A-C). In this context, the term "about" may be construed to mean 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids beyond the N-Terminus  
 20 and/or C-terminus of the above referenced TTL family domain polypeptide.

In preferred embodiments, the following TTL family domain is encompassed by the present invention:  
 EDIDTSADAVEDLTEAEWEDLTQQYYSLVHGDAFISNSRNYFSQCQALLNRIT  
 SVN PQTDIDGLRNIWIIKPAAKSRGRDIVCM DRV EEEILELAAADHPLSRDNKW  
 25 VVQKYIETPLLICDTKFDIRQWFLVTDWNPLTIWFYKESYLRFS TQRFS LDKL  
 DSAIHL CNNAVQKYLKNDVGRSPLLPAHNMWTSTRFQEYLQRQGRGAVWG  
 SVIYPSMKKAIAHAMKVAQDHVEPRKNSFELYGADFVLGRDFRPWLIEINSSP  
 TMHPSTPVTAQLCAQVQEDTIKVAVDRSCDI (SEQ ID NO:14). Polynucleotides encoding this polypeptide are also provided. The present invention also encompasses  
 30 the use of this BGS-42 TTL family domain polypeptide as an immunogenic and/or antigenic epitope as described elsewhere herein.



The BGS-42 polypeptide was also determined to comprise several conserved cysteines, at amino acid 155, 238, 347, 363, and/or 388 of SEQ ID No: 2 (Figures 1A-C). Conservation of cysteines at key amino acid residues is indicative of conserved structural features, which may correlate with conservation of protein function and/or activity.

The BGS-42 polypeptide was also determined to comprise a conserved cAMP-dependent protein kinase phosphorylation site located at amino acid 306 to amino acid 309 of SEQ ID NO:2 (Figures 1A-C). This cAMP-dependent protein kinase phosphorylation is found in other tubulin tyrosine ligase proteins and may be essential for either the activation or inhibition of tubulin tyrosine ligase activity.

In preferred embodiments, the present invention encompasses a polynucleotide lacking the initiating start codon, in addition to, the resulting encoded polypeptide of BGS-42. Specifically, the present invention encompasses the polynucleotide corresponding to nucleotides 156 thru 1775 of SEQ ID NO:1, and the polypeptide corresponding to amino acids 2 thru 541 of SEQ ID NO:2. Also encompassed are recombinant vectors comprising said encoding sequence, and host cells comprising said vector.

The present invention also encompasses a polynucleotide that encodes at least 424 contiguous amino acids of SEQ ID NO:2. Preferably such a polypeptide has tubulin ligase activity. The present invention also encompasses a polynucleotide that encodes about 424 contiguous amino acids of SEQ ID NO:2 wherein the term "about" may be construed to mean 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids beyond the N-Terminus and/or C-terminus of the above referenced polypeptide. The present invention also encompasses a polynucleotide comprising at least 1272 contiguous nucleotides of SEQ ID NO:1.

Expression profiling designed to measure the steady state mRNA levels encoding the BGS-42 polypeptide using SYBR green quantitative PCR (see Figure 8) showed predominately in the testis tissue. Expression of BGS-42 was also significantly expressed in brain, liver, and to a lesser extent, in other tissues.

Additional expression profiling designed to measure the steady state mRNA levels encoding the BGS-42 polypeptide using SYBR green quantitative PCR in normal versus tumor samples (see Figure 8) showed predominate differential

expression in lung tissue relative to lung tumor tissue, with lung tumor tissue showing a 25 fold decrease in BGS-42 expression relative to normal lung tissues.

Expanded expression profiling designed to measure the steady state mRNA levels encoding the BGS-42 polypeptide using TaqMan™ quantitative PCR (see Figure 10) showed BGS-42 was expressed predominately in the vas deferens. Expression of BGS-42 was also significantly expressed in lymph gland, pituitary, placenta, and to a lesser extent, in other tissues.

Additional expanded expression profiling data was also performed on mRNA isoalted from several testis tumors using TaqMan™ quantitative PCR (see Figure 11) showed that the testis specific expression of BGS-42 was lost in testis tumor tissue. The observation that BGS-42 is specifically downregulated in tumor samples suggests BGS-42 may play a role in tumor suppression, either directly or indirectly. Thus agonists directed against the BGS-42 polynucleotide or polypeptide would be useful for the treatment of tumors, particularly lung and testis tumors. In addition, BGS-42 polynucleotides may be useful as a diagnostic in identifying potential proliferative and/or tumor tissues relative to normal tissues, particularly in the detection of lung and testis tumors or proliferative conditions. Such a diagnostic would utilize the observed loss of BGS-42 expression in lung and/or testis tumor tissue as an indicia of proliferative state relative to the detectable expression in normal lung and/or testis tissues. Moreover, such a diagnostic may be useful for the detection of other tumor types by comparing BGS-42 expression in normal versus test tissue with loss of BGS-42 in the test tissue being diagnostic of a proliferative condition.

Based upon the strong homology to members of the tubulin tyrosine ligase protein family, the BGS-42 polypeptide is expected to share at least some biological activity with tubulin tyrosine ligase proteins (including, but limited to HOTTTL, TTL\_PIG, TTLH\_Hu, and TTLL\_Hu), and more preferably tubulin tyrosine ligase proteins found within testis, lymph node, pituitary, placenta, and/or vas deferens cells and tissues, in addition to the tubulin tyrosine ligase proteins referenced elsewhere herein or otherwise known in the art.

The present invention is also directed to the upstream, non-coding, promoter sequence of the BGS-42 gene (Figure 7A-B; SEQ ID NO:27). The polynucleotide sequence contains a sequence of 2241 nucleotides with nucleotides -2057 to -1 of

SEQ ID NO:27 representing the upstream promoter sequence. Analysis of the BGS-42 promoter region led to the identification of three CpG islands located from about nucleotide -1968 to about -1746; from about nucleotide -1232 to about -936; and/or from about nucleotide -727 to about -470 of SEQ ID NO:27 as determined using the algorithm described by D.T and P.A.J., PNAS 99(6):3740-5 (2002).

Recent evidence suggests the pattern of DNA methylation at CpG islands around promoter regions are often altered in cancer cells and that a correlation can be established between hyper-methylation within a promoter and lack of gene expression (Baylin et al., 2001). The silencing of gene expression is due to the recruitment of histone deacetylase to the methylated DNA. This can have the same effect as other types of tumor suppressor gene inactivation such as loss of homozygosity. The BGS-42 promoter region does indeed possess potential CpG beginning at positions -727, -1232 and -1968 relative to the ATG at +1. The altered methylation patterns within these regions might be operating to decrease the transcriptional activity at the BGS42 locus and hence decrease the steady-state RNA levels in proliferative tissues. The latter mechanism could explain the observed decrease in BGS-42 expression in lung and testis tumor tissues.

Therefore, the upstream, non-coding, promoter sequence of the BGS-42 gene from -2057 to -1 (Figure 7A-B; SEQ ID NO:27) is useful for decreasing the expression of a gene sequence in cells, particularly proliferative cells, when located upstream of a gene in a vector sequence. Alternatively, the location of one or more of the CpG islands identified in the BGS-42 promoter sequence are useful for decreasing the expression of a gene sequence in cells, particularly proliferative cells, when located upstream of a gene in a vector sequence.

The identification of three CpG islands within the BGS-42 promoter sequence, in combination with the observed decrease in BGS-42 expression in proliferative tissues suggests a role for BGS42 in the etiology of human cancers and that biological and pharmaceutical approaches to replacing BGS-42 function, which include, for example, the alteration of DNA methylation patterns within the BGS-42 promoter (e.g., deletion or alteration of CpG islands in the BGs-42 promoter to decrease the level of methylation), and/or the inhibition of histone deacetylase recruitment may represent a powerful new strategy for slowing tumor progression. Genomic scale

approaches to finding other genes that upon inhibition, restore BGS-42 expression and small molecule inhibitors to those gene products may have utility in the treatment of testicular and/or lung cancers. In addition, monitoring the attenuation of BGS42 gene expression may also provide a marker for tumor aggressiveness and have predictive value in determining therapeutic approaches.

The BGS-42 polynucleotides and polypeptides of the present invention, including agonists and/or fragments thereof, have uses that include modulating cellular proliferation and/or differentiation activity, in various cells, tissues, and organisms, and particularly in mammalian testis, lymph node, pituitary, placenta, and/or vas deferens cells and tissues, preferably human tissue.

The strong homology to human tubulin tyrosine ligase proteins, combined with the predominate localized expression in vas deferens/testis tissue suggests the potential utility for BGS-42 polynucleotides and polypeptides in treating, diagnosing, prognosing, and/or preventing testicular, in addition to reproductive disorders.

In preferred embodiments, BGS-42 polynucleotides and polypeptides including agonists and fragments thereof, have uses which include treating, diagnosing, prognosing, and/or preventing the following, non-limiting, diseases or disorders of the testis: spermatogenesis, infertility, Klinefelter's syndrome, XX male, epididymitis, genital warts, germinal cell aplasia, cryptorchidism, varicocele, immotile cilia syndrome, and viral orchitis. The BGS-42 polynucleotides and polypeptides including agonists and fragments thereof, may also have uses related to modulating testicular development, embryogenesis, reproduction, and in ameliorating, treating, and/or preventing testicular proliferative disorders (e.g., cancers, which include, for example, choriocarcinoma, Nonseminoma, seminoma, and testicular germ cell tumors).

Likewise, the predominate localized expression in testis tissue also emphasizes the potential utility for BGS-42 polynucleotides and polypeptides in treating, diagnosing, prognosing, and/or preventing metabolic diseases and disorders which include the following, not limiting examples: premature puberty, incomplete puberty, Kallman syndrome, Cushing's syndrome, hyperprolactinemia, hemochromatosis, congenital adrenal hyperplasia, FSH deficiency, and granulomatous disease, for example.

This gene product may also be useful in assays designed to identify binding agents, as such agents (antagonists) are useful as male contraceptive agents. The testes are also a site of active gene expression of transcripts that is expressed, particularly at low levels, in other tissues of the body. Therefore, this gene product may be expressed  
5 in other specific tissues or organs where it may play related functional roles in other processes, such as hematopoiesis, inflammation, bone formation, and kidney function, to name a few possible target indications.

The observed loss of BGS-42 expression in testis tumors relative to normal testis tissue suggests loss of the BGS-42 polypeptide may play a critical role in the  
10 development of a transformed phenotype leading to the development of cancers and/or a proliferative condition, either directly or indirectly. Alternatively, the loss of BGS-42 polypeptide expression may play a protective role and could be down regulated in response to a cancerous or proliferative phenotype (e.g., as a component of cell cycle regulation to inhibit further cellular damage that may be caused by  
15 proliferation). Whether loss of BGS-42 plays a role in directing transformation, or plays the role of protecting cells in response to a transformed phenotype, its role in testis tumors is likely to be enhanced. Therefore, agonists of the BGS-42 polypeptide may be useful in the treatment, amelioration, and/or prevention of a variety of proliferative conditions, including, but not limited to testis, in addition to other  
20 cancers or proliferative conditions.

The strong homology to human tubulin tyrosine ligase proteins, combined with the localized expression in lung tissue suggests the BGS-42 polynucleotides and polypeptides may be useful in treating, diagnosing, prognosing, and/or preventing pulmonary diseases and disorders which include the following, not limiting examples:  
25 ARDS, emphysema, cystic fibrosis, interstitial lung disease, chronic obstructive pulmonary disease, bronchitis, lymphangioliomyomatosis, pneumonitis, eosinophilic pneumonias, granulomatosis, pulmonary infarction, pulmonary fibrosis, pneumoconiosis, alveolar hemorrhage, neoplasms, lung abscesses, empyema, and increased susceptibility to lung infections (e.g., immunocompromised, HIV, etc.), for  
30 example.

Moreover, polynucleotides and polypeptides, including fragments and/or antagonists thereof, have uses which include, directly or indirectly, treating,

preventing, diagnosing, and/or prognosing the following, non-limiting, pulmonary infections: pneumonia, bacterial pneumonia, viral pneumonia (for example, as caused by Influenza virus, Respiratory syncytial virus, Parainfluenza virus, Adenovirus, Cocksackievirus, Cytomegalovirus, Herpes simplex virus, Hantavirus, etc.),

5 mycobacteria pneumonia (for example, as caused by *Mycobacterium tuberculosis*, etc.) mycoplasma pneumonia, fungal pneumonia (for example, as caused by *Pneumocystis carinii*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Candida* sp., *Cryptococcus neoformans*, *Aspergillus* sp., *Zygomycetes*, etc.), Legionnaires' Disease, Chlamydia pneumonia, aspiration pneumonia, *Nocardia* sp.

10 Infections, parasitic pneumonia (for example, as caused by *Strongyloides*, *Toxoplasma gondii*, etc.) necrotizing pneumonia, in addition to any other pulmonary disease and/or disorder (e.g., non-pneumonia) implicated by the causative agents listed above or elsewhere herein.

The observed loss of BGS-42 expression in lung tumors relative to normal

15 lung tissue suggests loss of the BGS-42 polypeptide may play a critical role in the development of a transformed phenotype leading to the development of cancers and/or a proliferative condition, either directly or indirectly. Alternatively, the loss of BGS-42 polypeptide expression may play a protective role and could be down regulated in response to a cancerous or proliferative phenotype (e.g., as a component

20 of cell cycle regulation to inhibit further cellular damage that may be caused by proliferation). Whether loss of BGS-42 plays a role in directing transformation, or plays the role of protecting cells in response to a transformed phenotype, its role in lung tumors is likely to be enhanced. Therefore, agonists of the BGS-42 polypeptide may be useful in the treatment, amelioration, and/or prevention of a variety of

25 proliferative conditions, including, but not limited to lung, in addition to other cancers or proliferative conditions.

The strong homology to human tubulin tyrosine ligase proteins, combined with the predominate localized expression in lymph node tissue suggests the BGS-42 polynucleotides and polypeptides may be useful in treating, diagnosing, prognosing,

30 and/or preventing immune diseases and/or disorders. Representative uses are described in the "Immune Activity", and "Infectious Disease" sections below, and elsewhere herein. Briefly, the strong expression in immune tissue indicates a role in

regulating the proliferation; survival; differentiation; and/or activation of hematopoietic cell lineages, including blood stem cells.

The BGS-42 polypeptide may also be useful as a preventative agent for immunological disorders including arthritis, asthma, immunodeficiency diseases such as AIDS, leukemia, rheumatoid arthritis, granulomatous disease, inflammatory bowel disease, sepsis, acne, neutropenia, neutrophilia, psoriasis, hypersensitivities, such as T-cell mediated cytotoxicity; immune reactions to transplanted organs and tissues, such as host-versus-graft and graft-versus-host diseases, or autoimmunity disorders, such as autoimmune infertility, lense tissue injury, demyelination, systemic lupus erythematosus, drug induced hemolytic anemia, rheumatoid arthritis, Sjogren's disease, and scleroderma. The BGS-42 polypeptide may be useful for modulating cytokine production, antigen presentation, or other processes, such as for boosting immune responses, etc.

Moreover, the protein may represent a secreted factor that influences the differentiation or behavior of other blood cells, or that recruits hematopoietic cells to sites of injury. Thus, this gene product is thought to be useful in the expansion of stem cells and committed progenitors of various blood lineages, and in the differentiation and/or proliferation of various cell types. Furthermore, the protein may also be used to determine biological activity, raise antibodies, as tissuemarkers, to isolate cognate ligands or receptors, to identify agents that modulate their interactions, in addition to its use as a nutritional supplement. Protein, as well as, antibodies directed against the protein may show utility as a tumor marker and/or immunotherapy targets for the above listed tissues.

The strong homology to human tubulin tyrosine ligase proteins, combined with the localized expression in pituitary gland tissue suggests the BGS-42 polynucleotides and polypeptides may be useful in treating, diagnosing, prognosing, and/or preventing endocrine diseases and/or disorders, which include, but are not limited to, the following: aberrant growth hormone synthesis and/or secretion, aberrant prolactin synthesis and/or secretion, aberrant luteinizing hormone synthesis and/or secretion, aberrant follicle-stimulating hormone synthesis and/or secretion, aberrant thyroid-stimulating hormone synthesis and/or secretion, aberrant adrenocorticotropin synthesis and/or secretion, aberrant vasopressin secretion,

aberrant oxytocin secretion, aberrant growth, aberrant lactation, aberrant sexual characteristic development, aberrant testosterone synthesis and/or secretion, aberrant estrogen synthesis and/or secretion, aberrant water homeostasis, hypogonadism, Addison's disease, hypothyroidism, Cushing's disease, agromegaly, gigantism, 5 lethargy, osteoporosis, aberrant calcium homeostasis, aberrant potassium homeostasis, reproductive disorders, and developmental disorders.

The strong homology to human tubulin tyrosine ligase proteins, combined with the localized expression in placenta tissue suggests the BGS-42 polynucleotides and polypeptides may be useful in treating, diagnosing, prognosing, and/or preventing 10 female reproductive diseases and/or disorders, particularly pre-term labor.

The BGS-42 polypeptide may also be useful as a preventative agent for immunological disorders including arthritis, asthma, immunodeficiency diseases such as AIDS, leukemia, rheumatoid arthritis, granulomatous disease, inflammatory bowel disease, sepsis, acne, neutropenia, neutrophilia, psoriasis, hypersensitivities, such as 15 T-cell mediated cytotoxicity; immune reactions to transplanted organs and tissues, such as host-versus-graft and graft-versus-host diseases, or autoimmunity disorders, such as autoimmune infertility, lense tissue injury, demyelination, systemic lupus erythematosus, drug induced hemolytic anemia, rheumatoid arthritis, Sjogren's disease, and scleroderma. The BGS-42 polypeptide may be useful for modulating 20 cytokine production, antigen presentation, or other processes, such as for boosting immune responses, etc.

Moreover, BGS-42 polynucleotides and polypeptides, including fragments and agonists thereof, may have uses which include treating, diagnosing, prognosing, and/or preventing hyperproliferative disorders, particularly of the reproductive, 25 immune, endocrine, and metabolic system. Such disorders may include, for example, cancers, and metastasis.

The BGS-42 polynucleotides and polypeptides, including fragments and /or antagonists thereof, may have uses which include identification of modulators of BGS-42 function including antibodies (for detection or neutralization), naturally- 30 occurring modulators and small molecule modulators. Antibodies to domains of the BGS-42 protein could be used as diagnostic agents of reproductive and inflammatory conditions in patients, are useful in monitoring the activation of signal transduction



pathways, and can be used as a biomarker for the involvement of tubulin tyrosine ligase proteins in disease states, and in the evaluation of agonists of tubulin tyrosine ligase proteins in vivo.

BGS-42 polypeptides and polynucleotides have additional uses which include  
5 diagnosing diseases related to the over and/or under expression of BGS-42 by  
identifying mutations in the BGS-42 gene by using BGS-42 sequences as probes or by  
determining BGS-42 protein or mRNA expression levels. BGS-42 polypeptides may  
be useful for screening compounds that affect the activity of the protein. BGS-42  
peptides can also be used for the generation of specific antibodies and as bait in yeast  
10 two hybrid screens to find proteins the specifically interact with BGS-42 (described  
elsewhere herein).

Although it is believed the encoded polypeptide may share at least some  
biological activities with tubulin tyrosine ligase proteins, a number of methods of  
determining the exact biological function of this clone are either known in the art or  
15 are described elsewhere herein. Briefly, the function of this clone may be determined  
by applying microarray methodology. Nucleic acids corresponding to the BGS-42  
polynucleotides, in addition to, other clones of the present invention, may be arrayed  
on microchips for expression profiling. Depending on which polynucleotide probe is  
used to hybridize to the slides, a change in expression of a specific gene may provide  
20 additional insight into the function of this gene based upon the conditions being  
studied. For example, an observed increase or decrease in expression levels when the  
polynucleotide probe used comes from diseased testis cells or tissue, as compared to,  
normal tissue might indicate a function in modulating reproductive function, for  
example. In the case of BGS-42, testis, lymph node, pituitary gland, and/or placenta  
25 tissue should be used, for example, to extract RNA to prepare the probe.

In addition, the function of the protein may be assessed by applying  
quantitative PCR methodology, for example. Real time quantitative PCR would  
provide the capability of following the expression of the BGS-42 gene throughout  
development, for example. Quantitative PCR methodology requires only a nominal  
30 amount of tissue from each developmentally important step is needed to perform such  
experiments. Therefore, the application of quantitative PCR methodology to refining  
the biological function of this polypeptide is encompassed by the present invention. In

the case of BGS-42, a disease correlation related to BGS-42 may be made by comparing the mRNA expression level of BGS-42 in normal tissue, as compared to diseased tissue (particularly diseased tissue isolated from the following: testis, lymph node, pituitary gland, and/or placenta tissue). Significantly higher or lower levels of BGS-42 expression in the diseased tissue may suggest BGS-42 plays a role in disease progression, and antagonists against BGS-42 polypeptides would be useful therapeutically in treating, preventing, and/or ameliorating the disease. Alternatively, significantly higher or lower levels of BGS-42 expression in the diseased tissue may suggest BGS-42 plays a defensive role against disease progression, and agonists of BGS-42 polypeptides may be useful therapeutically in treating, preventing, and/or ameliorating the disease. Also encompassed by the present invention are quantitative PCR probes corresponding to the polynucleotide sequence provided as SEQ ID NO:1 (Figures 1A-C).

The function of the protein may also be assessed through complementation assays in yeast. For example, in the case of the BGS-42, transforming yeast deficient in tubulin tyrosine ligase activity, for example, and assessing their ability to grow would provide convincing evidence the BGS-42 polypeptide has tubulin tyrosine ligase activity. Additional assay conditions and methods that may be used in assessing the function of the polynucleotides and polypeptides of the present invention are known in the art, some of which are disclosed elsewhere herein.

Alternatively, the biological function of the encoded polypeptide may be determined by disrupting a homologue of this polypeptide in Mice and/or rats and observing the resulting phenotype. Such knock-out experiments are known in the art, some of which are disclosed elsewhere herein.

Moreover, the biological function of this polypeptide may be determined by the application of antisense and/or sense methodology and the resulting generation of transgenic mice and/or rats. Expressing a particular gene in either sense or antisense orientation in a transgenic mouse or rat could lead to respectively higher or lower expression levels of that particular gene. Altering the endogenous expression levels of a gene can lead to the observation of a particular phenotype that can then be used to derive indications on the function of the gene. The gene can be either over-expressed or under expressed in every cell of the organism at all times using a strong ubiquitous

promoter, or it could be expressed in one or more discrete parts of the organism using a well characterized tissue-specific promoter (e.g., tubulin tyrosine ligase-tissue specific promoter), or it can be expressed at a specified time of development using an inducible and/or a developmentally regulated promoter.

5           In the case of BGS-42 transgenic mice or rats, if no phenotype is apparent in normal growth conditions, observing the organism under diseased conditions (reproductive, immune, endocrine, and metabolic disorders, in addition to cancers, etc.) may lead to understanding the function of the gene. Therefore, the application of antisense and/or sense methodology to the creation of transgenic mice or rats to refine  
10       the biological function of the polypeptide is encompassed by the present invention.

          In preferred embodiments, the following N-terminal BGS-42 deletion polypeptides are encompassed by the present invention: M1-S541, A2-S541, S3-S541, S4-S541, I5-S541, L6-S541, K7-S541, W8-S541, V9-S541, V10-S541, S11-S541, H12-S541, Q13-S541, S14-S541, C15-S541, S16-S541, R17-S541, S18-S541,  
15       S19-S541, R20-S541, S21-S541, K22-S541, P23-S541, R24-S541, D25-S541, Q26-S541, R27-S541, E28-S541, E29-S541, A30-S541, G31-S541, S32-S541, S33-S541, D34-S541, L35-S541, S36-S541, S37-S541, R38-S541, Q39-S541, D40-S541, A41-S541, E42-S541, N43-S541, A44-S541, E45-S541, A46-S541, K47-S541, L48-S541, R49-S541, G50-S541, L51-S541, P52-S541, G53-S541, Q54-S541, L55-S541, V56-S541,  
20       D57-S541, I58-S541, A59-S541, C60-S541, K61-S541, V62-S541, C63-S541, Q64-S541, A65-S541, Y66-S541, L67-S541, G68-S541, Q69-S541, L70-S541, E71-S541, H72-S541, E73-S541, D74-S541, I75-S541, D76-S541, T77-S541, S78-S541, A79-S541, D80-S541, A81-S541, V82-S541, E83-S541, D84-S541, L85-S541, T86-S541, E87-S541, A88-S541, E89-S541, W90-S541, E91-S541, D92-S541, L93-S541,  
25       T94-S541, Q95-S541, Q96-S541, Y97-S541, Y98-S541, S99-S541, L100-S541, V101-S541, H102-S541, G103-S541, D104-S541, A105-S541, F106-S541, I107-S541, S108-S541, N109-S541, S110-S541, R111-S541, N112-S541, Y113-S541, F114-S541, S115-S541, Q116-S541, C117-S541, Q118-S541, A119-S541, L120-S541, L121-S541, N122-S541, R123-S541, I124-S541, T125-S541, S126-S541,  
30       V127-S541, N128-S541, P129-S541, Q130-S541, T131-S541, D132-S541, I133-S541, D134-S541, G135-S541, L136-S541, R137-S541, N138-S541, I139-S541, W140-S541, I141-S541, I142-S541, K143-S541, P144-S541, A145-S541, A146-

S541, K147-S541, S148-S541, R149-S541, G150-S541, R151-S541, D152-S541,  
 I153-S541, V154-S541, C155-S541, M156-S541, D157-S541, R158-S541, V159-  
 S541, E160-S541, E161-S541, I162-S541, L163-S541, E164-S541, L165-S541,  
 A166-S541, A167-S541, A168-S541, D169-S541, H170-S541, P171-S541, L172-  
 5 S541, S173-S541, R174-S541, D175-S541, N176-S541, K177-S541, W178-S541,  
 V179-S541, V180-S541, Q181-S541, K182-S541, Y183-S541, I184-S541, E185-  
 S541, T186-S541, P187-S541, L188-S541, L189-S541, I190-S541, C191-S541,  
 D192-S541, T193-S541, K194-S541, F195-S541, D196-S541, I197-S541, R198-  
 S541, Q199-S541, W200-S541, F201-S541, L202-S541, V203-S541, T204-S541,  
 10 D205-S541, W206-S541, N207-S541, P208-S541, L209-S541, T210-S541, I211-  
 S541, W212-S541, F213-S541, Y214-S541, K215-S541, E216-S541, S217-S541,  
 Y218-S541, L219-S541, R220-S541, F221-S541, S222-S541, T223-S541, Q224-  
 S541, R225-S541, F226-S541, S227-S541, L228-S541, D229-S541, K230-S541,  
 L231-S541, D232-S541, S233-S541, A234-S541, I235-S541, H236-S541, L237-  
 15 S541, C238-S541, N239-S541, N240-S541, A241-S541, V242-S541, Q243-S541,  
 K244-S541, Y245-S541, L246-S541, K247-S541, N248-S541, D249-S541, V250-  
 S541, G251-S541, R252-S541, S253-S541, P254-S541, L255-S541, L256-S541,  
 P257-S541, A258-S541, H259-S541, N260-S541, M261-S541, W262-S541, T263-  
 S541, S264-S541, T265-S541, R266-S541, F267-S541, Q268-S541, E269-S541,  
 20 Y270-S541, L271-S541, Q272-S541, R273-S541, Q274-S541, G275-S541, R276-  
 S541, G277-S541, A278-S541, V279-S541, W280-S541, G281-S541, S282-S541,  
 V283-S541, I284-S541, Y285-S541, P286-S541, S287-S541, M288-S541, K289-  
 S541, K290-S541, A291-S541, I292-S541, A293-S541, H294-S541, A295-S541,  
 M296-S541, K297-S541, V298-S541, A299-S541, Q300-S541, D301-S541, H302-  
 25 S541, V303-S541, E304-S541, P305-S541, R306-S541, K307-S541, N308-S541,  
 S309-S541, F310-S541, E311-S541, L312-S541, Y313-S541, G314-S541, A315-  
 S541, D316-S541, F317-S541, V318-S541, L319-S541, G320-S541, R321-S541,  
 D322-S541, F323-S541, R324-S541, P325-S541, W326-S541, L327-S541, I328-  
 S541, E329-S541, I330-S541, N331-S541, S332-S541, S333-S541, P334-S541,  
 30 T335-S541, M336-S541, H337-S541, P338-S541, S339-S541, T340-S541, P341-  
 S541, V342-S541, T343-S541, A344-S541, Q345-S541, L346-S541, C347-S541,  
 A348-S541, Q349-S541, V350-S541, Q351-S541, E352-S541, D353-S541, T354-

S541, I355-S541, K356-S541, V357-S541, A358-S541, V359-S541, D360-S541,  
 R361-S541, S362-S541, C363-S541, D364-S541, I365-S541, G366-S541, N367-  
 S541, F368-S541, E369-S541, L370-S541, L371-S541, W372-S541, R373-S541,  
 Q374-S541, P375-S541, V376-S541, V377-S541, E378-S541, P379-S541, P380-  
 5 S541, P381-S541, F382-S541, S383-S541, G384-S541, S385-S541, D386-S541,  
 L387-S541, C388-S541, V389-S541, A390-S541, G391-S541, V392-S541, S393-  
 S541, V394-S541, R395-S541, R396-S541, A397-S541, R398-S541, R399-S541,  
 Q400-S541, V401-S541, L402-S541, P403-S541, V404-S541, C405-S541, N406-  
 S541, L407-S541, K408-S541, A409-S541, S410-S541, A411-S541, S412-S541,  
 10 L413-S541, L414-S541, D415-S541, A416-S541, Q417-S541, P418-S541, L419-  
 S541, K420-S541, A421-S541, R422-S541, G423-S541, P424-S541, S425-S541,  
 A426-S541, M427-S541, P428-S541, D429-S541, P430-S541, A431-S541, Q432-  
 S541, G433-S541, P434-S541, P435-S541, S436-S541, P437-S541, A438-S541,  
 L439-S541, Q440-S541, R441-S541, D442-S541, L443-S541, G444-S541, L445-  
 15 S541, K446-S541, E447-S541, E448-S541, K449-S541, G450-S541, L451-S541,  
 P452-S541, L453-S541, A454-S541, L455-S541, L456-S541, A457-S541, P458-  
 S541, L459-S541, R460-S541, G461-S541, A462-S541, A463-S541, E464-S541,  
 S465-S541, G466-S541, G467-S541, A468-S541, A469-S541, Q470-S541, P471-  
 S541, T472-S541, R473-S541, T474-S541, K475-S541, A476-S541, A477-S541,  
 20 G478-S541, K479-S541, V480-S541, E481-S541, L482-S541, P483-S541, A484-  
 S541, C485-S541, P486-S541, C487-S541, R488-S541, H489-S541, V490-S541,  
 D491-S541, S492-S541, Q493-S541, A494-S541, P495-S541, N496-S541, T497-  
 S541, G498-S541, V499-S541, P500-S541, V501-S541, A502-S541, Q503-S541,  
 P504-S541, A505-S541, K506-S541, S507-S541, W508-S541, D509-S541, P510-  
 25 S541, N511-S541, Q512-S541, L513-S541, N514-S541, A515-S541, H516-S541,  
 P517-S541, L518-S541, E519-S541, P520-S541, V521-S541, L522-S541, R523-  
 S541, G524-S541, L525-S541, K526-S541, T527-S541, A528-S541, E529-S541,  
 G530-S541, A531-S541, L532-S541, R533-S541, P534-S541, and/or P535-S541 of  
 SEQ ID NO:2. Polynucleotide sequences encoding these polypeptides are also  
 30 provided. The present invention also encompasses the use of these N-terminal BGS-  
 42 deletion polypeptides as immunogenic and/or antigenic epitopes as described  
 elsewhere herein.

In preferred embodiments, the following C-terminal BGS-42 deletion polypeptides are encompassed by the present invention: M1-S541, M1-G540, M1-K539, M1-G538, M1-G537, M1-P536, M1-P535, M1-P534, M1-R533, M1-L532, M1-A531, M1-G530, M1-E529, M1-A528, M1-T527, M1-K526, M1-L525, M1-G524, M1-R523, M1-L522, M1-V521, M1-P520, M1-E519, M1-L518, M1-P517, M1-H516, M1-A515, M1-N514, M1-L513, M1-Q512, M1-N511, M1-P510, M1-D509, M1-W508, M1-S507, M1-K506, M1-A505, M1-P504, M1-Q503, M1-A502, M1-V501, M1-P500, M1-V499, M1-G498, M1-T497, M1-N496, M1-P495, M1-A494, M1-Q493, M1-S492, M1-D491, M1-V490, M1-H489, M1-R488, M1-C487, M1-P486, M1-C485, M1-A484, M1-P483, M1-L482, M1-E481, M1-V480, M1-K479, M1-G478, M1-A477, M1-A476, M1-K475, M1-T474, M1-R473, M1-T472, M1-P471, M1-Q470, M1-A469, M1-A468, M1-G467, M1-G466, M1-S465, M1-E464, M1-A463, M1-A462, M1-G461, M1-R460, M1-L459, M1-P458, M1-A457, M1-L456, M1-L455, M1-A454, M1-L453, M1-P452, M1-L451, M1-G450, M1-K449, M1-E448, M1-E447, M1-K446, M1-L445, M1-G444, M1-L443, M1-D442, M1-R441, M1-Q440, M1-L439, M1-A438, M1-P437, M1-S436, M1-P435, M1-P434, M1-G433, M1-Q432, M1-A431, M1-P430, M1-D429, M1-P428, M1-M427, M1-A426, M1-S425, M1-P424, M1-G423, M1-R422, M1-A421, M1-K420, M1-L419, M1-P418, M1-Q417, M1-A416, M1-D415, M1-L414, M1-L413, M1-S412, M1-A411, M1-S410, M1-A409, M1-K408, M1-L407, M1-N406, M1-C405, M1-V404, M1-P403, M1-L402, M1-V401, M1-Q400, M1-R399, M1-R398, M1-A397, M1-R396, M1-R395, M1-V394, M1-S393, M1-V392, M1-G391, M1-A390, M1-V389, M1-C388, M1-L387, M1-D386, M1-S385, M1-G384, M1-S383, M1-F382, M1-P381, M1-P380, M1-P379, M1-E378, M1-V377, M1-V376, M1-P375, M1-Q374, M1-R373, M1-W372, M1-L371, M1-L370, M1-E369, M1-F368, M1-N367, M1-G366, M1-I365, M1-D364, M1-C363, M1-S362, M1-R361, M1-D360, M1-V359, M1-A358, M1-V357, M1-K356, M1-I355, M1-T354, M1-D353, M1-E352, M1-Q351, M1-V350, M1-Q349, M1-A348, M1-C347, M1-L346, M1-Q345, M1-A344, M1-T343, M1-V342, M1-P341, M1-T340, M1-S339, M1-P338, M1-H337, M1-M336, M1-T335, M1-P334, M1-S333, M1-S332, M1-N331, M1-I330, M1-E329, M1-I328, M1-L327, M1-W326, M1-P325, M1-R324, M1-F323, M1-D322, M1-R321, M1-G320, M1-L319, M1-V318, M1-F317, M1-D316, M1-A315, M1-G314, M1-Y313,

M1-L312, M1-E311, M1-F310, M1-S309, M1-N308, M1-K307, M1-R306, M1-P305,  
 M1-E304, M1-V303, M1-H302, M1-D301, M1-Q300, M1-A299, M1-V298, M1-  
 K297, M1-M296, M1-A295, M1-H294, M1-A293, M1-I292, M1-A291, M1-K290,  
 M1-K289, M1-M288, M1-S287, M1-P286, M1-Y285, M1-I284, M1-V283, M1-S282,  
 5 M1-G281, M1-W280, M1-V279, M1-A278, M1-G277, M1-R276, M1-G275, M1-  
 Q274, M1-R273, M1-Q272, M1-L271, M1-Y270, M1-E269, M1-Q268, M1-F267,  
 M1-R266, M1-T265, M1-S264, M1-T263, M1-W262, M1-M261, M1-N260, M1-  
 H259, M1-A258, M1-P257, M1-L256, M1-L255, M1-P254, M1-S253, M1-R252,  
 M1-G251, M1-V250, M1-D249, M1-N248, M1-K247, M1-L246, M1-Y245, M1-  
 10 K244, M1-Q243, M1-V242, M1-A241, M1-N240, M1-N239, M1-C238, M1-L237,  
 M1-H236, M1-I235, M1-A234, M1-S233, M1-D232, M1-L231, M1-K230, M1-  
 D229, M1-L228, M1-S227, M1-F226, M1-R225, M1-Q224, M1-T223, M1-S222,  
 M1-F221, M1-R220, M1-L219, M1-Y218, M1-S217, M1-E216, M1-K215, M1-  
 Y214, M1-F213, M1-W212, M1-I211, M1-T210, M1-L209, M1-P208, M1-N207,  
 15 M1-W206, M1-D205, M1-T204, M1-V203, M1-L202, M1-F201, M1-W200, M1-  
 Q199, M1-R198, M1-I197, M1-D196, M1-F195, M1-K194, M1-T193, M1-D192,  
 M1-C191, M1-I190, M1-L189, M1-L188, M1-P187, M1-T186, M1-E185, M1-I184,  
 M1-Y183, M1-K182, M1-Q181, M1-V180, M1-V179, M1-W178, M1-K177, M1-  
 N176, M1-D175, M1-R174, M1-S173, M1-L172, M1-P171, M1-H170, M1-D169,  
 20 M1-A168, M1-A167, M1-A166, M1-L165, M1-E164, M1-L163, M1-I162, M1-E161,  
 M1-E160, M1-V159, M1-R158, M1-D157, M1-M156, M1-C155, M1-V154, M1-  
 I153, M1-D152, M1-R151, M1-G150, M1-R149, M1-S148, M1-K147, M1-A146,  
 M1-A145, M1-P144, M1-K143, M1-I142, M1-I141, M1-W140, M1-I139, M1-N138,  
 M1-R137, M1-L136, M1-G135, M1-D134, M1-I133, M1-D132, M1-T131, M1-  
 25 Q130, M1-P129, M1-N128, M1-V127, M1-S126, M1-T125, M1-I124, M1-R123,  
 M1-N122, M1-L121, M1-L120, M1-A119, M1-Q118, M1-C117, M1-Q116, M1-  
 S115, M1-F114, M1-Y113, M1-N112, M1-R111, M1-S110, M1-N109, M1-S108,  
 M1-I107, M1-F106, M1-A105, M1-D104, M1-G103, M1-H102, M1-V101, M1-  
 L100, M1-S99, M1-Y98, M1-Y97, M1-Q96, M1-Q95, M1-T94, M1-L93, M1-D92,  
 30 M1-E91, M1-W90, M1-E89, M1-A88, M1-E87, M1-T86, M1-L85, M1-D84, M1-  
 E83, M1-V82, M1-A81, M1-D80, M1-A79, M1-S78, M1-T77, M1-D76, M1-I75,  
 M1-D74, M1-E73, M1-H72, M1-E71, M1-L70, M1-Q69, M1-G68, M1-L67, M1-

Y66, M1-A65, M1-Q64, M1-C63, M1-V62, M1-K61, M1-C60, M1-A59, M1-I58, M1-D57, M1-V56, M1-L55, M1-Q54, M1-G53, M1-P52, M1-L51, M1-G50, M1-R49, M1-L48, M1-K47, M1-A46, M1-E45, M1-A44, M1-N43, M1-E42, M1-A41, M1-D40, M1-Q39, M1-R38, M1-S37, M1-S36, M1-L35, M1-D34, M1-S33, M1-S32, 5 M1-G31, M1-A30, M1-E29, M1-E28, M1-R27, M1-Q26, M1-D25, M1-R24, M1-P23, M1-K22, M1-S21, M1-R20, M1-S19, M1-S18, M1-R17, M1-S16, M1-C15, M1-S14, M1-Q13, M1-H12, M1-S11, M1-V10, M1-V9, M1-W8, and/or M1-K7 of SEQ ID NO:2. Polynucleotide sequences encoding these polypeptides are also provided. The present invention also encompasses the use of these C-terminal BGS-42 deletion 10 polypeptides as immunogenic and/or antigenic epitopes as described elsewhere herein.

Alternatively, preferred polypeptides of the present invention may comprise polypeptide sequences corresponding to, for example, internal regions of the BGS-42 polypeptide (e.g., any combination of both N- and C- terminal BGS-42 polypeptide 15 deletions) of SEQ ID NO:2. For example, internal regions could be defined by the equation: amino acid NX to amino acid CX, wherein NX refers to any N-terminal deletion polypeptide amino acid of BGS-42 (SEQ ID NO:2), and where CX refers to any C-terminal deletion polypeptide amino acid of BGS-42 (SEQ ID NO:2). Polynucleotides encoding these polypeptides are also provided. The present invention 20 also encompasses the use of these polypeptides as an immunogenic and/or antigenic epitope as described elsewhere herein.

The present invention also encompasses immunogenic and/or antigenic epitopes of the BGS-42 polypeptide.

The BGS-42 polypeptides of the present invention were determined to 25 comprise several phosphorylation sites based upon the Motif algorithm (Genetics Computer Group, Inc.). The phosphorylation of such sites may regulate some biological activity of the BGS-42 polypeptide. For example, phosphorylation at specific sites may be involved in regulating the proteins ability to associate or bind to other molecules (e.g., proteins, ligands, substrates, DNA, etc.). In the present case, 30 phosphorylation may modulate the ability of the BGS-42 polypeptide to associate with other polypeptides, particularly cognate ligand for BGS-42, or its ability to modulate certain cellular signal pathways.



The BGS-42 polypeptide was predicted to comprise seven PKC phosphorylation sites using the Motif algorithm (Genetics Computer Group, Inc.). In vivo, protein kinase C exhibits a preference for the phosphorylation of serine or threonine residues. The PKC phosphorylation sites have the following consensus pattern: [ST]-x-[RK], where S or T represents the site of phosphorylation and 'x' an intervening amino acid residue. Additional information regarding PKC phosphorylation sites can be found in Woodget J.R., Gould K.L., Hunter T., Eur. J. Biochem. 161:177-184(1986), and Kishimoto A., Nishiyama K., Nakanishi H., Uratsuji Y., Nomura H., Takeyama Y., Nishizuka Y., J. Biol. Chem.... 260:12492-12499(1985); which are hereby incorporated by reference herein.

In preferred embodiments, the following PKC phosphorylation site polypeptides are encompassed by the present invention: QSCSRSSRSKPRD (SEQ ID NO:31), GSSDLSSRQDAEN (SEQ ID NO:32), YLRFSTQRFSLDK (SEQ ID NO:33), HNMWTSTRFQEYL (SEQ ID NO:34), SVIYPSMKKAIAH (SEQ ID NO:35), QVQEDTIKVAVDR (SEQ ID NO:36), and/or CVAGVSVRRARRQ (SEQ ID NO:37). Polynucleotides encoding these polypeptides are also provided. The present invention also encompasses the use of the BGS-42 PKC phosphorylation site polypeptides as immunogenic and/or antigenic epitopes as described elsewhere herein.

The BGS-42 polypeptide was predicted to comprise seven casein kinase II phosphorylation sites using the Motif algorithm (Genetics Computer Group, Inc.). Casein kinase II (CK-2) is a protein serine/threonine kinase whose activity is independent of cyclic nucleotides and calcium. CK-2 phosphorylates many different proteins. The substrate specificity [1] of this enzyme can be summarized as follows:

- (1) Under comparable conditions Ser is favored over Thr.;
- (2) An acidic residue (either Asp or Glu) must be present three residues from the C-terminal of the phosphate acceptor site;
- (3) Additional acidic residues in positions +1, +2, +4, and +5 increase the phosphorylation rate. Most physiological substrates have at least one acidic residue in these positions;
- (4) Asp is preferred to Glu as the provider of acidic determinants;
- (5) A basic residue at the N-terminal of the acceptor site decreases the phosphorylation rate, while an acidic one will increase it.

A consensus pattern for casein kinase II phosphorylations site is as follows: [ST]-x(2)-[DE], wherein 'x' represents any amino acid, and S or T is the phosphorylation site.

Additional information specific to casein kinase II phosphorylation sites may  
5 be found in reference to the following publication: Pinna L.A., Biochim. Biophys. Acta 1054:267-284(1990); which is hereby incorporated herein in its entirety.

In preferred embodiments, the following casein kinase II phosphorylation site polypeptide is encompassed by the present invention: SSDLSSRQDAENAE (SEQ ID NO:38), HEDIDTSADAVEDL (SEQ ID NO:39), AVELDLTEAEWEDLT (SEQ ID  
10 NO:40), SVN PQTDIDGLRNI (SEQ ID NO:41), LLICDTKFDIRQWF (SEQ ID NO:42), EPPPFSGSDLCVAG (SEQ ID NO:43), and/or LKASASLLDAQPLK (SEQ ID NO:44). Polynucleotides encoding these polypeptides are also provided. The present invention also encompasses the use of this casein kinase II phosphorylation site polypeptide as an immunogenic and/or antigenic epitope as described elsewhere  
15 herein.

The BGS-42 polypeptide was predicted to comprise one cAMP- and cGMP-dependent protein kinase phosphorylation site using the Motif algorithm (Genetics Computer Group, Inc.). There has been a number of studies relative to the specificity of cAMP- and cGMP-dependent protein kinases. Both types of kinases appear to  
20 share a preference for the phosphorylation of serine or threonine residues found close to at least two consecutive N-terminal basic residues.

A consensus pattern for cAMP- and cGMP-dependent protein kinase phosphorylation sites is as follows: [RK](2)-x-[ST], wherein "x" represents any amino acid, and S or T is the phosphorylation site.

Additional information specific to cAMP- and cGMP-dependent protein kinase phosphorylation sites may be found in reference to the following publication: Fremisco J.R., Glass D.B., Krebs E.G, J. Biol. Chem.. 255:4240-4245(1980); Glass D.B., Smith S.B., J. Biol. Chem.. 258:14797-14803(1983); and Glass D.B., El-Maghrabi M.R., Pilkis S.J., J. Biol. Chem.. 261:2987-2993(1986); which is hereby  
25  
30 incorporated herein in its entirety.

In preferred embodiments, the following cAMP- and cGMP-dependent protein kinase phosphorylation site polypeptide is encompassed by the present invention:

DHVEPRKNSFELYG (SEQ ID NO:45). Polynucleotides encoding this polypeptide are also provided. The present invention also encompasses the use of this cAMP- and cGMP-dependent protein kinase phosphorylation site polypeptide as an immunogenic and/or antigenic epitope as described elsewhere herein.

5           The PMN29 polypeptide was predicted to comprise six N-myristoylation sites using the Motif algorithm (Genetics Computer Group, Inc.). An appreciable number of eukaryotic proteins are acylated by the covalent addition of myristate (a C14-saturated fatty acid) to their N-terminal residue via an amide linkage. The sequence specificity of the enzyme responsible for this modification, myristoyl CoA:protein N-  
10 myristoyl transferase (NMT), has been derived from the sequence of known N-myristoylated proteins and from studies using synthetic peptides. The specificity seems to be the following: i.) The N-terminal residue must be glycine; ii.) In position 2, uncharged residues are allowed; iii.) Charged residues, proline and large hydrophobic residues are not allowed; iv.) In positions 3 and 4, most, if not all,  
15 residues are allowed; v.) In position 5, small uncharged residues are allowed (Ala, Ser, Thr, Cys, Asn and Gly). Serine is favored; and vi.) In position 6, proline is not allowed.

          A consensus pattern for N-myristoylation is as follows: G-{EDRKHPFYW}-  
x(2)-[STAGCN]-{P}, wherein 'x' represents any amino acid, and G is the N-  
20 myristoylation site.

          Additional information specific to N-myristoylation sites may be found in reference to the following publication: Towler D.A., Gordon J.I., Adams S.P., Glaser L., Annu. Rev. Biochem. 57:69-99(1988); and Grand R.J.A., Biochem. J. 258:625-638(1989); which is hereby incorporated herein in its entirety.

25           In preferred embodiments, the following N-myristoylation site polypeptides are encompassed by the present invention: QRQGRGAVWGSVIYPS (SEQ ID NO:46), PPPFSGSDLCVAGVSV (SEQ ID NO:47), LKEEKGLPLALLAPLR (SEQ ID NO:48), LAPLRGAAESGGAAQP (SEQ ID NO:49), QAPNTGVPVAQPAKSW (SEQ ID NO:50), and/or EPVLRGLKTAEGALRP (SEQ ID NO:51). Polynucleotides  
30 encoding these polypeptides are also provided. The present invention also encompasses the use of these N-myristoylation site polypeptides as immunogenic and/or antigenic epitopes as described elsewhere herein.

The present invention encompasses the identification of compounds and drugs which stimulate BGS-42 on the one hand (i.e., agonists) and which inhibit the function of BGS-42 on the other hand (i.e., antagonists). In general, such screening procedures involve providing appropriate cells which express the polypeptide of the present invention on the surface thereof. Such cells may include, for example, cells from mammals, yeast, *Drosophila* or *E. coli*. In a preferred embodiment, a polynucleotide encoding the polypeptide of the present invention may be employed to transfect cells to thereby express the BGS-42 polypeptide. The expressed polypeptide may then be contacted with a test compound to observe binding, stimulation or inhibition of a functional response.

In confirmation that the BGS-42 polypeptide is a tyrosine ligase, molecular modeling of BGS-42 determined that it contained a distinct structural domain exemplified by the catalytic domain of glutathione synthetase, a member of the peptide synthases ADP-forming enzymes (acid-D-amino acid ligases), E.C.6.3.2.-. Based upon the sequence, structure, motifs, known binding signature sequences, and homology to known tyrosine ligase proteins, the inventors have determined that BGS-42 contains a novel tubulin-tyrosine ligase domain and have ascribed the BGS-42 polypeptide as having tubulin-tyrosine ligase (E.C.6.3.2.25) activity(s) and cellular and systemic regulatory function(s).

The three dimensional crystallographic structures for several ADP forming enzymes from E.C.6.3.2.-, acid-D-amino acid ligases have been reported and are deposited into the Protein Data Bank (Bernstein et. al., 1977 & Berman et. al., 2000). X-ray structures have been determined for E.C.6.3.2.1, Pantoate-beta-alanine ligase; E.C.6.3.2.3, Glutathione synthase; E.C.6.3.2.4, D-alanine-D-alanine ligase; E.C.6.3.2.8, UDP-N-acetylmuramate-alanine ligase, E.C.6.3.2.9, UDP-N-acetylmuramoylalanine-D-glutamate ligase; E.C.6.3.2.15, UDP-N-acetylmuramoylalanine-D-glutamyl-2,6-diaminopimelate-D-alanyl; E.C.6.3.2.17, folylpolyglutamate synthase; E.C.6.3.2.19, Ubiquitin-protein ligase. Comparison of the three-dimensional structures (Dideberg & Bertrand, 1998) for several of these family members identified a structural motif which forms the ATP binding site. Dideberg and Bertrand (Trends Biochem. Sci. 23:57-58, 1998) describe structural differences for the ADP-forming peptide synthetases and highlighted distinct

structural families including the glutathione synthetases. In their analyses they concluded that tyrosine ligase, E.C.6.3.2.25, an enzyme involved in tubulin modification, most likely belongs to the glutathione synthetase ADP-forming synthetase family, E.C.6.3.2.3.

5           The glutathione synthetase structure is a structural prototype for the ADP-forming synthetase family (E.C.6.3.2.3). Glutathione synthetase is ubiquitous among organisms and catalyzes the ligation of gamma-L-glutamyl-L-cysteine and glycine with the aid of ATP in the presence of magnesium ion(Mg<sup>2+</sup>). The structure of glutathione synthetase (Hara et al., Biochemistry. 35:11967-11974. 1996.) was  
10 obtained from the Protein Data Bank (PDB) and has the PDB code 1GSA. The ATP binding fold of glutathione synthetase contains two motifs. The first motif consists of a four-stranded beta-sheet with two coupled helices and a small flexible loop. The second motif consists of a five-stranded beta sheet and a large flexible loop that is positioned at the edge of the sheet and the first sheet motif. The flexible loops extend  
15 over substrates bound in the active site and moves from the “open” position to the “closed” position when substrates are bound to the active site. Several amino acids are conserved in these ADP-forming enzymes and include amino acids found in direct contact with the nucleotide ADP. For glutathione synthetase K160, G167, Q198, Y200, D208, D273, E281, N283 represent several residues that interact with ADP in  
20 the active site via interactions with the nucleotide base, sugar, alpha-phosphate and beta-phosphate.

Homology models are useful when there is no experimental information available on the protein of interest. A three dimensional model can be constructed on the basis of the known structure of a homologous protein (Greer *et. al.*, 1991, Lesk, *et. al.*, 1992, Levitt, 1992, Cardozo, *et. al.*, 1995, Sali, *et. al.*, 1995).  
25

Those of skill in the art will understand that a homology model is constructed on the basis of first identifying a template, or, protein of known structure which is similar to the protein without known structure. This can be accomplished by through pairwise alignment of sequences using such programs as FASTA (Pearson, *et. al.*  
30 1990) and BLAST (Altschul, *et. al.*, 1990). In cases where sequence similarity is high (greater than 30 %) these pairwise comparison methods may be adequate. Likewise, multiple sequence alignments or profile-based methods can be used to align a query

sequence to an alignment of multiple (structurally and biochemically) related proteins. When the sequence similarity is low, more advanced techniques are used such as fold recognition (protein threading; Hendlich, *et. al.*, 1990, Koppensteiner *et. al.* 2000, Sippl & Weitckus 1992, Sippl 1993), where the compatibility of a particular sequence with the three dimensional fold of a potential template protein is gauged on the basis of a knowledge-based potential. Following the initial sequence alignment, the query template can be optimally aligned by manual manipulation or by incorporation of other features (motifs, secondary structure predictions, and allowed sequence conservation). Next, structurally conserved regions can be identified and are used to construct the core secondary structure (Levitt, 1992, Sali, *et. al.*, 1995) elements in the three dimensional model. Variable regions, called “unconserved regions” and loops can be added using knowledge-based techniques. The complete model with variable regions and loops can be refined performing forcefield calculations (Sali, *et. al.*, 1995, Cardozo, *et. al.*, 1995).

For BGS-42 a hand generated sequence alignment based upon the three structural motifs identified by Dideberg and Bertrand (1998) produced 13% overall identity (for amino acids M1 to R361) of BGS-42 aligned with the sequence human glutathione synthetase, E.C. number 6.3.2.3 (Hara *et al.*, 1998), (Protein Data Bank code 1GSA). The alignment of BGS-42 with PDB entry 1GSA is set forth in Figure 13. In this invention, the homology model of BGS-42 was derived from the sequence alignment set forth in Figure 13. An overall atomic model including plausible sidechain orientations was generated using the program LOOK (Levitt, 1992). The three dimensional model for BGS-42 is defined by the set of structure coordinates as set forth in Table IV and is shown in Figure 14 rendered by backbone secondary structures.

The term “structure coordinates” refers to Cartesian coordinates generated from the building of a homology model.

Those of skill in the art will understand that a set of structure coordinates for a protein is a relative set of points that define a shape in three dimensions. Thus, it is possible that an entirely different set of coordinates could define a similar or identical shape. Moreover, slight variations in the individual coordinates, as emanate from generation of similar homology models using different alignment templates (*i.e.*, other

than the structure coordinates of 1GSA), and/or using different methods in generating the homology model, will have minor effects on the overall shape. Variations in coordinates may also be generated because of mathematical manipulations of the structure coordinates. For example, the structure coordinates set forth in Table IV  
5 could be manipulated by fractionalization of the structure coordinates; integer additions or subtractions to sets of the structure coordinates, inversion of the structure coordinates or any combination of the above.

Various computational analyses are therefore necessary to determine whether a molecule or a portion thereof is sufficiently similar to all or parts of BGS-42  
10 described above as to be considered the same. Such analyses may be carried out in current software applications, such as INSIGHTII (Accelrys Inc., San Diego, CA) version 2000 as described in the User's Guide, online ([www.accelrys.com](http://www.accelrys.com)) or software applications available in the SYBYL software suite (Tripos Inc., St. Louis, MO).

Using the superimposition tool in the program INSIGHTII comparisons can be  
15 made between different structures and different conformations of the same structure. The procedure used in INSIGHTII to compare structures is divided into four steps: 1) load the structures to be compared; 2) define the atom equivalencies in these structures; 3) perform a fitting operation; and 4) analyze the results. Each structure is identified by a name. One structure is identified as the target (i.e., the fixed structure);  
20 the second structure (i.e., moving structure) is identified as the source structure. Since atom equivalency within INSIGHTII is defined by user input, for the purpose of this invention, equivalent atoms are defined as protein backbone atoms (N, C $\alpha$ , C and O) for all conserved residues between the two structures being compared. Also, only rigid fitting operations were considered. When a rigid fitting method is used, the  
25 working structure is translated and rotated to obtain an optimum fit with the target structure. The fitting operation uses an algorithm that computes the optimum translation and rotation to be applied to the moving structure, such that the root mean square difference of the fit over the specified pairs of equivalent atoms are an absolute minimum. This number, given in angstroms, is reported by INSIGHTII. For the  
30 purpose of this invention, any homology model of a BGS-42 that has a root mean square deviation of conserved residue backbone atoms (N, C $\alpha$ , C, O) of less than about 4.0, 3.0, 2.0, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 angstroms when

superimposed on the relevant backbone atoms described by structure coordinates listed in Table IV are considered identical.

5 The term "root mean square deviation" means the square root of the arithmetic mean of the squares of the deviations from the mean. It is a way to express the deviation or variation from a trend or object. For purposes of this invention, the "root mean square deviation" defines the variation in the backbone of a protein from the relevant portion of the backbone of BGS-42 as defined by the structure coordinates described herein.

10 This invention as embodied by the three-dimensional model enables the structure-based design of modulators of the biological function of BGS-42, as well as mutants with altered biological function and/or specificity.

The sequence alignment (Figure 13) used to provide a template for creation of the three-dimensional model of BGS-42 tyrosine ligase domain has 13% sequence identity between catalytic domain of BGS-42 and Escherichia coli glutathione synthetase, PDB code 1GSA. For the peptide synthases ADP-forming enzymes (acid-  
15 D-amino acid ligases), E.C.6.3.2.-, the functionally important residues are located in the active site and bind substrate and nucleotide, ATP. Residues of glutathione synthetase that interact with ADP in the active site via interactions with the nucleotide base, sugar, and phosphate groups include K160, G167, Q198, Y200, D208, D273,  
20 E281, N283. These residues are highlighted in the sequence alignment provided in Figure 13. The other active site residues are also highlighted in Figure 13 and are described by (Dideberg & Bertrand, 1998) as conserved hydrophobic residues in the structural motifs associated with the ATP active site. The following is a list of residues within the BGS-42 polypeptide sequence that correspond to the above  
25 mentioned active site residues of glutathione synthetase: K143 of SEQ ID NO:2 corresponds to K160, G150 of SEQ ID NO:2 corresponds to G167, Q181 of SEQ ID NO:2 corresponds to Q198, Y183 of SEQ ID NO:2 corresponds to Y200, D196 of SEQ ID NO:2 corresponds to D208, D316 of SEQ ID NO:2 corresponds to D273, E281 of SEQ ID NO:2 corresponds to E329, and N283 of SEQ ID NO:2 corresponds  
30 to N331. Clearly, as evidenced by the latter in addition to the 3D model of BGS-42, the active site residues required for tyrosine ligase activity are completely conserved between BGS42 and glutathione synthetase. This conservation suggests that a



structural model of the active site can be accurately created even though the overall identity for the domain is 13%.

Figure 14 shows the structure of the BGS-42 and has highlighted the active site side chains that are conserved and are homologous to those in the template glutathione synthetase, 1GSA. The structure of glutathione synthetase has two motifs with flexible loops that extend over the bound substrate. For BGS there is a large insertion, residues E216-W262 that cannot be aligned with any region of the template 1GSA. This loop region is not provided in the homology model as this region may form a large flexible loop that may interact with tubulin when it binds with the enzyme (BGS42). This potential flexible loop is consistent with and analagous to the two structural motifs found in glutathione synthetase.

This is a strong indication that the functional activity for this domain of BGS-42 is a tyrosine ligase (E.C.6.3.2.-) and most probably a tubulin-tyrosine ligase (E.C.6.3.2.25). In addition the three-dimensional model of BGS-42 (Figure 14) shows that the active site residues are located in exactly the same three-dimensional position as the 1GSA structure which allows for these residues to interact with ATP in using the same binding mode.

The structure coordinates of a BGS-42 homology model portion thereof are stored in a machine-readable storage medium. Such data may be used for a variety of purposes, such as drug discovery and target prioritization and validation.

Accordingly, in one embodiment of this invention is provided a machine-readable data storage medium comprising a data storage material encoded with the structure coordinates set forth in Table IV.

For the first time, the present invention permits the use, through homology modeling based upon the sequence of BGS-42 (Figures 13 and 14) of structure-based or rational drug design techniques to design, select, and synthesizes chemical entities that are capable of modulating the biological function of BGS-42. Comparison of the BGS-42 homology model with the structures of other amino acid ligases enable the use of rational or structure based drug design methods to design, select or synthesize specific chemical modulators of BGS-42.

Accordingly, the present invention is also directed to the entire sequence in Figures 1A-C, or any portion thereof for the purpose of generating a homology model for the purpose of three dimensional structure-based drug designs.

For purposes of this invention, we include mutants or homologues of the sequence in Figures 1A-C, or any portion thereof. In a preferred embodiment, the mutants or homologues have at least 25% identity, more preferably 50% identity, more preferably 75% identity, and most preferably 90% identity to the amino acid residues in Figures 1A-C.

The three-dimensional model structure of the BGS-42 will also provide methods for identifying modulators of biological function. Various methods or combination thereof can be used to identify these compounds.

Structure coordinates of the active site region defined above can also be used to identify structural and chemical features. Identified structural or chemical features can then be employed to design or select compounds as potential BGS-42 modulators. By structural and chemical features it is meant to include, but is not limited to, van der Waals interactions, hydrogen bonding interactions, charge interaction, hydrophobic interactions, and dipole interaction. Alternatively, or in conjunction, the three-dimensional structural model can be employed to design or select compounds as potential BGS-42 modulators. Compounds identified as potential BGS-42 modulators can then be synthesized and screened in an assay characterized by binding of a test compound to the BGS-42, or in characterizing BGS-42 deactivation in the presence of a small molecule. Examples of assays useful in screening of potential BGS-42 modulators include, but are not limited to, screening *in silico*, *in vitro* assays and high throughput assays. Finally, these methods may also involve modifying or replacing one or more amino acids from BGS-42 according to Table IV.

However, as will be understood by those of skill in the art upon this disclosure, other structure based design methods can be used. Various computational structure based design methods have been disclosed in the art.

For example, a number of computer modeling systems are available in which the sequence of the BGS-42 and the BGS-42 structure (i.e., atomic coordinates of BGS-42 and/or the atomic coordinates of the active site region as provided in Table IV) can be input. The computer system then generates the structural details of one or

more these regions in which a potential BGS-42 modulator binds so that complementary structural details of the potential modulators can be determined. Design in these modeling systems is generally based upon the compound being capable of physically and structurally associating with BGS-42. In addition, the  
5 compound must be able to assume a conformation that allows it to associate with BGS-42. Some modeling systems estimate the potential inhibitory or binding effect of a potential BGS-42 modulator prior to actual synthesis and testing.

Methods for screening chemical entities or fragments for their ability to associate with a given protein target are well known. Often these methods begin by  
10 visual inspection of the binding site on the computer screen. Selected fragments or chemical entities are then positioned in one or more positions and orientations within the active site region in BGS-42. Molecular docking is accomplished using software such as INSIGHTII, ICM (Molsoft LLC, La Jolla, CA), and SYBYL, following by energy minimization and molecular dynamics with standard molecular mechanic  
15 forcefields such as CHARMM and MMFF. Examples of computer programs which assist in the selection of chemical fragment or chemical entities useful in the present invention include, but are not limited to, GRID (Goodford, 1985), AUTODOCK (Goodsell, 1990), and DOCK (Kuntz *et. al.* 1982).

Alternatively, compounds may be designed de novo using either an empty  
20 active site or optionally including some portion of a known inhibitor. Methods of this type of design include, but are not limited to LUDI (Bohm 1992), LeapFrog (Tripos Associates, St. Louis MO) and DOCK (Kuntz *et. al.*, 1982). Programs such as DOCK (Kuntz *et. al.* 1982) can be used with the atomic coordinates from the homology model to identify potential ligands from databases or virtual databases which  
25 potentially bind the in the active site region, and which may therefore be suitable candidates for synthesis and testing. The computer programs may utilize a combination of the following steps: a.) Selection of fragments or chemical entities from a database and then positioning the chemical entity in one or more orientations within the BGS-42 active site defined by residues K143, G150, Q181, Y183, D196,  
30 D316, E281, and N283 or portion thereof; b.) Characterization of the structural and chemical features of the chemical entity and active site including van der Waals interactions, hydrogen bonding interactions, charge interaction, hydrophobic bonding

interaction, and dipole interactions; c.) Search databases for molecular fragments which can be joined to or replace the docked chemical entity and spatially fit into regions defined by the said BGS-42 active site site defined by residues K143, G150, Q181, Y183, D196, D316, E281, and N283 or portion thereof; and/or d.) Evaluate the  
5 docked chemical entity and fragments using a combination of scoring schemes which account for van der Waals interactions, hydrogen bonding interactions, charge interaction, hydrophobic interactions

Databases that may be used include ACD (Molecular Designs Limited), Aldrich (Aldrich Chemical Company), NCI (National Cancer Institute),  
10 Maybridge(Maybridge Chemical Company Ltd), CCDC (Cambridge Crystallographic Data Center), CAST (Chemical Abstract Service), Derwent (Derwent Information Limited).

Upon selection of preferred chemical entities or fragments, their relationship to each other and BGS-42 can be visualized and then assembled into a single potential  
15 modulator. Programs useful in assembling the individual chemical entities include, but are not limited to SYBYL and LeapFrog (Tripos Associates, St. Louis MO), LUDI (Bohm 1992) as well as 3D Database systems (Martin 1992).

Additionally, the three-dimensional homology model of BGS-42 will aid in the design of mutants with altered biological activity. Site directed mutagenesis can  
20 be used to generate proteins with similar or varying degrees of biological activity compared to native BGS-42. This invention also relates to the generation of mutants or homologs of BGS-42. It is clear that molecular modeling using the three dimensional structure coordinates set forth in Table IV and visualization of the BGS-42 models, Figure 14 can be utilized to design homologs or mutant polypeptides of  
25 BGS-42 that have similar or altered biological activities, function or reactivities.

In preferred embodiments, the following BGS-42 active site domain amino acid substitutions are encompassed by the present invention: wherein K143 is substituted with either an A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; wherein P144 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S,  
30 T, V, W, or Y; wherein A145 is substituted with either a C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein A146 is substituted with either a C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein K147 is substituted with either an

A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; wherein S148 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; wherein R149 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein G150 is substituted with either an A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein R151 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein D152 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein I153 is substituted with either an A, C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein V154 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; wherein C155 is substituted with either an A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein M156 is substituted with either an A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, or Y; wherein D157 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein R158 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein V159 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; wherein E160 is substituted with either an A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein E161 is substituted with either an A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein I162 is substituted with either an A, C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein L163 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein E164 is substituted with either an A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein L165 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein A166 is substituted with either a C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein A167 is substituted with either a C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein A168 is substituted with either a C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein D169 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein H170 is substituted with either an A, C, D, E, F, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein P171 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; wherein L172 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein S173 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; wherein R174 is substituted with

either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein D175 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein N176 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; wherein K177 is substituted with either an A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; wherein W178 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or Y; wherein V179 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; wherein V180 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; wherein Q181 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; wherein K182 is substituted with either an A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; wherein Y183 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; wherein I184 is substituted with either an A, C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein E185 is substituted with either an A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein T186 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y; wherein P187 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; wherein L188 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein L189 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein I190 is substituted with either an A, C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein C191 is substituted with either an A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein D192 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein T193 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y; wherein K194 is substituted with either an A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; wherein F195 is substituted with either an A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein D196 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein I197 is substituted with either an A, C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein R198 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein Q199 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; wherein W200 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or Y;

wherein F201 is substituted with either an A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein L202 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein V203 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; wherein T204 is substituted with either an  
5 A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y; wherein D205 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein W206 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or Y; wherein N207 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; wherein P208 is substituted with either an A, C, D, E, F,  
10 G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; wherein L209 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein T210 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y; wherein I211 is substituted with either an A, C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein W212 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P,  
15 Q, R, S, T, V, or Y; wherein F213 is substituted with either an A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein Y214 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; wherein K215 is substituted with either an A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; wherein E216 is substituted with either an A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;  
20 wherein S217 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; wherein Y218 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; wherein L219 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein R220 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein F221 is substituted  
25 with either an A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein S222 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; wherein T223 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y; wherein Q224 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; wherein R225 is substituted with either an A, C,  
30 D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein F226 is substituted with either an A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein S227 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y;

wherein L228 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein D229 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein K230 is substituted with either an A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; wherein L231 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein D232 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein S233 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; wherein A234 is substituted with either a C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein I235 is substituted with either an A, C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein H236 is substituted with either an A, C, D, E, F, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein L237 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein C238 is substituted with either an A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein N239 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; wherein N240 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; wherein A241 is substituted with either a C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein V242 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; wherein Q243 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; wherein K244 is substituted with either an A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; wherein Y245 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; wherein L246 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein K247 is substituted with either an A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; wherein N248 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; wherein D249 is substituted with either an A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein V250 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; wherein G251 is substituted with either an A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein R252 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein S253 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; wherein P254 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, Q,



R, S, T, V, W, or Y; wherein L255 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein L256 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein P257 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; wherein A258 is substituted with either a C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein H259 is substituted with either an A, C, D, E, F, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein N260 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; wherein M261 is substituted with either an A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, or Y; wherein W262 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or Y; wherein T263 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y; wherein S264 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; wherein T265 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y; wherein R266 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein F267 is substituted with either an A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein Q268 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; wherein E269 is substituted with either an A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein Y270 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; wherein L271 is substituted with either an A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; wherein Q272 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; wherein R273 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein Q274 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; wherein G275 is substituted with either an A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein R276 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; wherein G277 is substituted with either an A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein A278 is substituted with either a C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; wherein V279 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; wherein W280 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or Y; wherein G281 is substituted with either an A, C, D, E, F, H, I, K, L, M,

N, P, Q, R, S, T, V, W, or Y; wherein S282 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; and/or wherein V283 is substituted with either an A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y of SEQ ID NO:2, in addition to any combination thereof. The present invention also encompasses the  
 5 use of these BGS-42 active site domain amino acid substituted polypeptides as immunogenic and/or antigenic epitopes as described elsewhere herein.

In preferred embodiments, the following BGS-42 active site domain conservative amino acid substitutions are encompassed by the present invention: wherein K143 is substituted with either a R, or H; wherein P144 is a P; wherein A145  
 10 is substituted with either a G, I, L, M, S, T, or V; wherein A146 is substituted with either a G, I, L, M, S, T, or V; wherein K147 is substituted with either a R, or H; wherein S148 is substituted with either an A, G, M, or T; wherein R149 is substituted with either a K, or H; wherein G150 is substituted with either an A, M, S, or T; wherein R151 is substituted with either a K, or H; wherein D152 is substituted with an  
 15 E; wherein I153 is substituted with either an A, V, or L; wherein V154 is substituted with either an A, I, or L; wherein C155 is a C; wherein M156 is substituted with either an A, G, S, or T; wherein D157 is substituted with an E; wherein R158 is substituted with either a K, or H; wherein V159 is substituted with either an A, I, or L; wherein E160 is substituted with a D; wherein E161 is substituted with a D; wherein  
 20 I162 is substituted with either an A, V, or L; wherein L163 is substituted with either an A, I, or V; wherein E164 is substituted with a D; wherein L165 is substituted with either an A, I, or V; wherein A166 is substituted with either a G, I, L, M, S, T, or V; wherein A167 is substituted with either a G, I, L, M, S, T, or V; wherein A168 is substituted with either a G, I, L, M, S, T, or V; wherein D169 is substituted with an E;  
 25 wherein H170 is substituted with either a K, or R; wherein P171 is a P; wherein L172 is substituted with either an A, I, or V; wherein S173 is substituted with either an A, G, M, or T; wherein R174 is substituted with either a K, or H; wherein D175 is substituted with an E; wherein N176 is substituted with a Q; wherein K177 is substituted with either a R, or H; wherein W178 is either an F, or Y; wherein V179 is  
 30 substituted with either an A, I, or L; wherein V180 is substituted with either an A, I, or L; wherein Q181 is substituted with a N; wherein K182 is substituted with either a R, or H; wherein Y183 is either an F, or W; wherein I184 is substituted with either an

A, V, or L; wherein E185 is substituted with a D; wherein T186 is substituted with either an A, G, M, or S; wherein P187 is a P; wherein L188 is substituted with either an A, I, or V; wherein L189 is substituted with either an A, I, or V; wherein I190 is substituted with either an A, V, or L; wherein C191 is a C; wherein D192 is substituted with an E; wherein T193 is substituted with either an A, G, M, or S; wherein K194 is substituted with either a R, or H; wherein F195 is substituted with either a W, or Y; wherein D196 is substituted with an E; wherein I197 is substituted with either an A, V, or L; wherein R198 is substituted with either a K, or H; wherein Q199 is substituted with a N; wherein W200 is either an F, or Y; wherein F201 is substituted with either a W, or Y; wherein L202 is substituted with either an A, I, or V; wherein V203 is substituted with either an A, I, or L; wherein T204 is substituted with either an A, G, M, or S; wherein D205 is substituted with an E; wherein W206 is either an F, or Y; wherein N207 is substituted with a Q; wherein P208 is a P; wherein L209 is substituted with either an A, I, or V; wherein T210 is substituted with either an A, G, M, or S; wherein I211 is substituted with either an A, V, or L; wherein W212 is either an F, or Y; wherein F213 is substituted with either a W, or Y; wherein Y214 is either an F, or W; wherein K215 is substituted with either a R, or H; wherein E216 is substituted with a D; wherein S217 is substituted with either an A, G, M, or T; wherein Y218 is either an F, or W; wherein L219 is substituted with either an A, I, or V; wherein R220 is substituted with either a K, or H; wherein F221 is substituted with either a W, or Y; wherein S222 is substituted with either an A, G, M, or T; wherein T223 is substituted with either an A, G, M, or S; wherein Q224 is substituted with a N; wherein R225 is substituted with either a K, or H; wherein F226 is substituted with either a W, or Y; wherein S227 is substituted with either an A, G, M, or T; wherein L228 is substituted with either an A, I, or V; wherein D229 is substituted with an E; wherein K230 is substituted with either a R, or H; wherein L231 is substituted with either an A, I, or V; wherein D232 is substituted with an E; wherein S233 is substituted with either an A, G, M, or T; wherein A234 is substituted with either a G, I, L, M, S, T, or V; wherein I235 is substituted with either an A, V, or L; wherein H236 is substituted with either a K, or R; wherein L237 is substituted with either an A, I, or V; wherein C238 is a C; wherein N239 is substituted with a Q; wherein N240 is substituted with a Q; wherein A241 is substituted with either a G, I, L, M, S, T, or

V; wherein V242 is substituted with either an A, I, or L; wherein Q243 is substituted  
 with a N; wherein K244 is substituted with either a R, or H; wherein Y245 is either an  
 F, or W; wherein L246 is substituted with either an A, I, or V; wherein K247 is  
 substituted with either a R, or H; wherein N248 is substituted with a Q; wherein D249  
 5 is substituted with an E; wherein V250 is substituted with either an A, I, or L; wherein  
 G251 is substituted with either an A, M, S, or T; wherein R252 is substituted with  
 either a K, or H; wherein S253 is substituted with either an A, G, M, or T; wherein  
 P254 is a P; wherein L255 is substituted with either an A, I, or V; wherein L256 is  
 substituted with either an A, I, or V; wherein P257 is a P; wherein A258 is substituted  
 10 with either a G, I, L, M, S, T, or V; wherein H259 is substituted with either a K, or R;  
 wherein N260 is substituted with a Q; wherein M261 is substituted with either an A,  
 G, S, or T; wherein W262 is either an F, or Y; wherein T263 is substituted with either  
 an A, G, M, or S; wherein S264 is substituted with either an A, G, M, or T; wherein  
 T265 is substituted with either an A, G, M, or S; wherein R266 is substituted with  
 15 either a K, or H; wherein F267 is substituted with either a W, or Y; wherein Q268 is  
 substituted with a N; wherein E269 is substituted with a D; wherein Y270 is either an  
 F, or W; wherein L271 is substituted with either an A, I, or V; wherein Q272 is  
 substituted with a N; wherein R273 is substituted with either a K, or H; wherein Q274  
 is substituted with a N; wherein G275 is substituted with either an A, M, S, or T;  
 20 wherein R276 is substituted with either a K, or H; wherein G277 is substituted with  
 either an A, M, S, or T; wherein A278 is substituted with either a G, I, L, M, S, T, or  
 V; wherein V279 is substituted with either an A, I, or L; wherein W280 is either an F,  
 or Y; wherein G281 is substituted with either an A, M, S, or T; wherein S282 is  
 substituted with either an A, G, M, or T; and/or wherein V283 is substituted with  
 25 either an A, I, or L of SEQ ID NO:2 in addition to any combination thereof. Other  
 suitable substitutions within the BGS-42 active site domain are encompassed by the  
 present invention and are referenced elsewhere herein. The present invention also  
 encompasses the use of these BGS-42 active site domain conservative amino acid  
 substituted polypeptides as immunogenic and/or antigenic epitopes as described  
 30 elsewhere herein.

Many polynucleotide sequences, such as EST sequences, are publicly  
 available and accessible through sequence databases. Some of these sequences are

related to SEQ ID NO: 1 and may have been publicly available prior to conception of the present invention. Preferably, such related polynucleotides are specifically excluded from the scope of the present invention. To list every related sequence would be cumbersome. Accordingly, preferably excluded from the present invention are one or more polynucleotides consisting of a nucleotide sequence described by the general formula of a-b, where a is any integer between 1 to 1824 of SEQ ID NO:1, b is an integer between 15 to 1838, where both a and b correspond to the positions of nucleotide residues shown in SEQ ID NO:1, and where b is greater than or equal to a+14.

In one embodiment, a BGS-42 polypeptide comprises a portion of the amino sequence depicted in Figures 1A-C. In another embodiment, a BGS-42 polypeptide comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids of the amino sequence depicted in Figures 1A-C. In further embodiments, the following BGS-42 polypeptide fragments are specifically excluded from the present invention: KWVVQKYIE (SEQ ID NO:15); RQWFLVTDWNPLT (SEQ ID NO:16); SFELYGADFV (SEQ ID NO:17); QWFLVTDWNPLT (SEQ ID NO:18); NIWIKPAAKSRGRDIVCMDRVE (SEQ ID NO:19); DNKWVVQKYIETP (SEQ ID NO:20); DTKFDIRQWFLVTDWNPLTIWIFYKESYLRFSQSLDKLDSAIHLCNN (SEQ ID NO:21); SSPTMHPSTPVTAQLCAQVQEDTIKV (SEQ ID NO:22); CDIGNFELLWRQP (SEQ ID NO:23); LPACPCRHVDSQAPNTGVPVAQPAKSWDPNQLNAHPLEPVLR (SEQ ID NO:24); and/or LKTAEGALRPPPGGKGS (SEQ ID NO:25).

25

TABLE I

Gene No.	CDNA Clone ID	ATCC Deposit No. Z and Date	Vector	NT SEQ ID. No. X	Total NT Seq of Clone	5' NT of Start Codon of ORF	3' NT of ORF	AA Seq ID No. Y	Total AA of ORF
1.	BGS-42	PTA-4454 06/12/02	pSport1	1	1838	153	1775	2	541

Table I summarizes the information corresponding to each "Gene No." described above. The nucleotide sequence identified as "NT SEQ ID NO:X" was

assembled from partially homologous ("overlapping") sequences obtained from the "cDNA clone ID" identified in Table I and, in some cases, from additional related DNA clones. The overlapping sequences were assembled into a single contiguous sequence of high redundancy (usually several overlapping sequences at each  
5 nucleotide position), resulting in a final sequence identified as SEQ ID NO:X.

The cDNA Clone ID was deposited on the date and given the corresponding deposit number listed in "ATCC Deposit No:Z and Date." "Vector" refers to the type of vector contained in the cDNA Clone ID.

"Total NT Seq. Of Clone" refers to the total number of nucleotides in the  
10 clone contig identified by "Gene No." The deposited clone may contain all or most of the sequence of SEQ ID NO:X. The nucleotide position of SEQ ID NO:X of the putative start codon (methionine) is identified as "5' NT of Start Codon of ORF."

The translated amino acid sequence, beginning with the methionine, is identified as "AA SEQ ID NO:Y" although other reading frames can also be easily  
15 translated using known molecular biology techniques. The polypeptides produced by these alternative open reading frames are specifically contemplated by the present invention.

The total number of amino acids within the open reading frame of SEQ ID NO:Y is identified as "Total AA of ORF".

20 SEQ ID NO:X (where X may be any of the polynucleotide sequences disclosed in the sequence listing) and the translated SEQ ID NO:Y (where Y may be any of the polypeptide sequences disclosed in the sequence listing) are sufficiently accurate and otherwise suitable for a variety of uses well known in the art and described further herein. For instance, SEQ ID NO:X is useful for designing nucleic  
25 acid hybridization probes that will detect nucleic acid sequences contained in SEQ ID NO:X or the cDNA contained in the deposited clone. These probes will also hybridize to nucleic acid molecules in biological samples, thereby enabling a variety of forensic and diagnostic methods of the invention. Similarly, polypeptides identified from SEQ ID NO:Y may be used, for example, to generate antibodies which bind specifically to  
30 proteins containing the polypeptides and the proteins encoded by the cDNA clones identified in Table I.

Nevertheless, DNA sequences generated by sequencing reactions can contain sequencing errors. The errors exist as misidentified nucleotides, or as insertions or deletions of nucleotides in the generated DNA sequence. The erroneously inserted or deleted nucleotides may cause frame shifts in the reading frames of the predicted amino acid sequence. In these cases, the predicted amino acid sequence diverges from the actual amino acid sequence, even though the generated DNA sequence may be greater than 99.9% identical to the actual DNA sequence (for example, one base insertion or deletion in an open reading frame of over 1000 bases).

Accordingly, for those applications requiring precision in the nucleotide sequence or the amino acid sequence, the present invention provides not only the generated nucleotide sequence identified as SEQ ID NO:1 and the predicted translated amino acid sequence identified as SEQ ID NO:2, but also a sample of plasmid DNA containing a cDNA of the invention deposited with the ATCC, as set forth in Table I. The nucleotide sequence of each deposited clone can readily be determined by sequencing the deposited clone in accordance with known methods. The predicted amino acid sequence can then be verified from such deposits. Moreover, the amino acid sequence of the protein encoded by a particular clone can also be directly determined by peptide sequencing or by expressing the protein in a suitable host cell containing the deposited cDNA, collecting the protein, and determining its sequence.

The present invention also relates to the genes corresponding to SEQ ID NO:1, SEQ ID NO:2, or the deposited clone. The corresponding gene can be isolated in accordance with known methods using the sequence information disclosed herein. Such methods include preparing probes or primers from the disclosed sequence and identifying or amplifying the corresponding gene from appropriate sources of genomic material.

Also provided in the present invention are species homologs, allelic variants, and/or orthologs. The skilled artisan could, using procedures well-known in the art, obtain the polynucleotide sequence corresponding to full-length genes (including, but not limited to the full-length coding region), allelic variants, splice variants, orthologs, and/or species homologues of genes corresponding to SEQ ID NO:1, SEQ ID NO:2, or a deposited clone, relying on the sequence from the sequences disclosed herein or the clones deposited with the ATCC. For example, allelic variants and/or species

homologues may be isolated and identified by making suitable probes or primers which correspond to the 5', 3', or internal regions of the sequences provided herein and screening a suitable nucleic acid source for allelic variants and/or the desired homologue.

5           The polypeptides of the invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

10           The polypeptides may be in the form of the protein, or may be a part of a larger protein, such as a fusion protein (see below). It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification, such as multiple histidine residues, or an additional sequence for stability during recombinant production.

15           The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of a polypeptide, can be substantially purified using techniques described herein or otherwise known in the art, such as, for example, by the one-step method described in Smith and Johnson, Gene 67:31-40 (1988). Polypeptides of the invention  
20 also can be purified from natural, synthetic or recombinant sources using protocols described herein or otherwise known in the art, such as, for example, antibodies of the invention raised against the full-length form of the protein.

          The present invention provides a polynucleotide comprising, or alternatively consisting of, the sequence identified as SEQ ID NO:1, and/or a cDNA provided in  
25 ATCC Deposit No. Z:. The present invention also provides a polypeptide comprising, or alternatively consisting of, the sequence identified as SEQ ID NO:2, and/or a polypeptide encoded by the cDNA provided in ATCC Deposit NO:Z. The present invention also provides polynucleotides encoding a polypeptide comprising, or alternatively consisting of the polypeptide sequence of SEQ ID NO:2, and/or a  
30 polypeptide sequence encoded by the cDNA contained in ATCC Deposit No:Z.

          Preferably, the present invention is directed to a polynucleotide comprising, or alternatively consisting of, the sequence identified as SEQ ID NO:1, and/or a cDNA



provided in ATCC Deposit No.: that is less than, or equal to, a polynucleotide sequence that is 5 mega basepairs, 1 mega basepairs, 0.5 mega basepairs, 0.1 mega basepairs, 50,000 basepairs, 20,000 basepairs, or 10,000 basepairs in length.

The present invention encompasses polynucleotides with sequences complementary to those of the polynucleotides of the present invention disclosed herein. Such sequences may be complementary to the sequence disclosed as SEQ ID NO:1, the sequence contained in a deposit, and/or the nucleic acid sequence encoding the sequence disclosed as SEQ ID NO:2.

The present invention also encompasses polynucleotides capable of hybridizing, preferably under reduced stringency conditions, more preferably under stringent conditions, and most preferably under highly stringent conditions, to polynucleotides described herein. Examples of stringency conditions are shown in Table II below: highly stringent conditions are those that are at least as stringent as, for example, conditions A-F; stringent conditions are at least as stringent as, for example, conditions G-L; and reduced stringency conditions are at least as stringent as, for example, conditions M-R.

TABLE II

Stringency Condition	Polynucleotide Hybrid±	Hybrid Length (bp) ‡	Hybridization Temperature and Buffer†	Wash Temperature and Buffer †
A	DNA:DNA	> or equal to 50	65°C; 1xSSC – or- 42°C; 1xSSC, 50% formamide	65°C; 0.3xSSC
B	DNA:DNA	< 50	Tb*; 1xSSC	Tb*; 1xSSC
C	DNA:RNA	> or equal to 50	67°C; 1xSSC – or- 45°C; 1xSSC, 50% formamide	67°C; 0.3xSSC
D	DNA:RNA	< 50	Td*; 1xSSC	Td*; 1xSSC
E	RNA:RNA	> or equal to 50	70°C; 1xSSC – or- 50°C; 1xSSC, 50% formamide	70°C; 0.3xSSC
F	RNA:RNA	< 50	Tf*; 1xSSC	Tf*; 1xSSC
G	DNA:DNA	> or equal to 50	65°C; 4xSSC – or- 45°C; 4xSSC, 50% formamide	65°C; 1xSSC
H	DNA:DNA	< 50	Th*; 4xSSC	Th*; 4xSSC

Stringency Condition	Polynucleotide Hybrid±	Hybrid Length (bp) ‡	Hybridization Temperature and Buffer†	Wash Temperature and Buffer †
I	DNA:RNA	> or equal to 50	67°C; 4xSSC – or- 45°C; 4xSSC, 50% formamide	67°C; 1xSSC
J	DNA:RNA	< 50	Tj*; 4xSSC	Tj*; 4xSSC
K	RNA:RNA	> or equal to 50	70°C; 4xSSC – or- 40°C; 6xSSC, 50% formamide	67°C; 1xSSC
L	RNA:RNA	< 50	TI*; 2xSSC	TI*; 2xSSC
M	DNA:DNA	> or equal to 50	50°C; 4xSSC – or- 40°C 6xSSC, 50% formamide	50°C; 2xSSC
N	DNA:DNA	< 50	Tn*; 6xSSC	Tn*; 6xSSC
O	DNA:RNA	> or equal to 50	55°C; 4xSSC – or- 42°C; 6xSSC, 50% formamide	55°C; 2xSSC
P	DNA:RNA	< 50	Tp*; 6xSSC	Tp*; 6xSSC
Q	RNA:RNA	> or equal to 50	60°C; 4xSSC – or- 45°C; 6xSSC, 50% formamide	60°C; 2xSSC
R	RNA:RNA	< 50	Tr*; 4xSSC	Tr*; 4xSSC

‡ - The “hybrid length” is the anticipated length for the hybridized region(s) of the hybridizing polynucleotides. When hybridizing a polynucleotide of unknown sequence, the hybrid is assumed to be that of the hybridizing polynucleotide of the present invention. When polynucleotides of known sequence are hybridized, the hybrid length can be determined by aligning the sequences of the polynucleotides and identifying the region or regions of optimal sequence complementarity. Methods of aligning two or more polynucleotide sequences and/or determining the percent identity between two polynucleotide sequences are well known in the art (e.g., MegAlign program of the DNA\*Star suite of programs, etc).

† - SSPE (1xSSPE is 0.15M NaCl, 10mM NaH<sub>2</sub>PO<sub>4</sub>, and 1.25mM EDTA, pH 7.4) can be substituted for SSC (1xSSC is 0.15M NaCl and 15mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes after hybridization is complete. The hybridizations and washes may additionally include 5X Denhardt's reagent, .5-1.0% SDS, 100ug/ml denatured, fragmented salmon sperm DNA, 0.5% sodium pyrophosphate, and up to 50% formamide.

\*T<sub>b</sub> – T<sub>r</sub>: The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature T<sub>m</sub> of the hybrids there T<sub>m</sub> is determined according to the following equations. For hybrids less than 18 base pairs in length,  $T_m(^{\circ}\text{C}) = 2(\# \text{ of A + T bases}) + 4(\# \text{ of G + C bases})$ . For  
5 hybrids between 18 and 49 base pairs in length,  $T_m(^{\circ}\text{C}) = 81.5 + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G+C}) - (600/N)$ , where N is the number of bases in the hybrid, and [Na<sup>+</sup>] is the concentration of sodium ions in the hybridization buffer ([Na<sup>+</sup>] for 1xSSC = .165 M).

± - The present invention encompasses the substitution of any one, or more  
10 DNA or RNA hybrid partners with either a PNA, or a modified polynucleotide. Such modified polynucleotides are known in the art and are more particularly described elsewhere herein.

Additional examples of stringency conditions for polynucleotide hybridization  
15 are provided, for example, in Sambrook, J., E.F. Fritsch, and T. Maniatis, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, chapters 9 and 11, and Current Protocols in Molecular Biology, 1995, F.M., Ausubel et al., eds, John Wiley and Sons, Inc., sections 2.10 and 6.3-6.4, which are hereby incorporated by reference herein.

20 Preferably, such hybridizing polynucleotides have at least 70% sequence identity (more preferably, at least 80% identity; and most preferably at least 90% or 95% identity) with the polynucleotide of the present invention to which they hybridize, where sequence identity is determined by comparing the sequences of the hybridizing polynucleotides when aligned so as to maximize overlap and identity  
25 while minimizing sequence gaps. The determination of identity is well known in the art, and discussed more specifically elsewhere herein.

The invention encompasses the application of PCR methodology to the polynucleotide sequences of the present invention, the clone deposited with the ATCC, and/or the cDNA encoding the polypeptides of the present invention. PCR  
30 techniques for the amplification of nucleic acids are described in US Patent No. 4, 683, 195 and Saiki et al., Science, 239:487-491 (1988). PCR, for example, may include the following steps, of denaturation of template nucleic acid (if double-

stranded), annealing of primer to target, and polymerization. The nucleic acid probed or used as a template in the amplification reaction may be genomic DNA, cDNA, RNA, or a PNA. PCR may be used to amplify specific sequences from genomic DNA, specific RNA sequence, and/or cDNA transcribed from mRNA. References for the general use of PCR techniques, including specific method parameters, include Mullis et al., Cold Spring Harbor Symp. Quant. Biol., 51:263, (1987), Ehrlich (ed), PCR Technology, Stockton Press, NY, 1989; Ehrlich et al., Science, 252:1643-1650, (1991); and "PCR Protocols, A Guide to Methods and Applications", Eds., Innis et al., Academic Press, New York, (1990).

#### 10 Polynucleotide and Polypeptide Variants

The present invention also encompasses variants (e.g., allelic variants, orthologs, etc.) of the polynucleotide sequence disclosed herein in SEQ ID NO:1, the complementary strand thereto, and/or the cDNA sequence contained in the deposited clone.

15 The present invention also encompasses variants of the polypeptide sequence, and/or fragments therein, disclosed in SEQ ID NO:2, a polypeptide encoded by the polynucleotide sequence in SEQ ID NO:1, and/or a polypeptide encoded by a cDNA in the deposited clone.

"Variant" refers to a polynucleotide or polypeptide differing from the polynucleotide or polypeptide of the present invention, but retaining essential properties thereof. Generally, variants are overall closely similar, and, in many regions, identical to the polynucleotide or polypeptide of the present invention.

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising, or alternatively consisting of, a polynucleotide having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding a BGS-42 related polypeptide having an amino acid sequence as shown in the sequence listing and described in SEQ ID NO:1 or the cDNA contained in ATCC deposit No:Z; (b) a nucleotide sequence encoding a mature BGS-42 related polypeptide having the amino acid sequence as shown in the sequence listing and described in SEQ ID NO:1 or the cDNA contained in ATCC deposit No:Z; (c) a nucleotide sequence encoding a biologically active fragment of a BGS-42 related polypeptide having an amino acid sequence shown in the sequence listing and described in SEQ ID NO:1 or the cDNA

contained in ATCC deposit No:Z; (d) a nucleotide sequence encoding an antigenic fragment of a BGS-42 related polypeptide having an amino acid sequence shown in the sequence listing and described in SEQ ID NO:1 or the cDNA contained in ATCC deposit No:Z; (e) a nucleotide sequence encoding a BGS-42 related polypeptide comprising the complete amino acid sequence encoded by a human cDNA plasmid contained in SEQ ID NO:1 or the cDNA contained in ATCC deposit No:Z; (f) a nucleotide sequence encoding a mature BGS-42 related polypeptide having an amino acid sequence encoded by a human cDNA plasmid contained in SEQ ID NO:1 or the cDNA contained in ATCC deposit No:Z; (g) a nucleotide sequence encoding a biologically active fragment of a BGS-42 related polypeptide having an amino acid sequence encoded by a human cDNA plasmid contained in SEQ ID NO:1 or the cDNA contained in ATCC deposit No:Z; (h) a nucleotide sequence encoding an antigenic fragment of a BGS-42 related polypeptide having an amino acid sequence encoded by a human cDNA plasmid contained in SEQ ID NO:1 or the cDNA contained in ATCC deposit No:Z; (I) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), or (h), above.

The present invention is also directed to polynucleotide sequences which comprise, or alternatively consist of, a polynucleotide sequence which is at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% identical to, for example, any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), or (h), above. Polynucleotides encoded by these nucleic acid molecules are also encompassed by the invention. In another embodiment, the invention encompasses nucleic acid molecules which comprise, or alternatively, consist of a polynucleotide which hybridizes under stringent conditions, or alternatively, under lower stringency conditions, to a polynucleotide in (a), (b), (c), (d), (e), (f), (g), or (h), above. Polynucleotides which hybridize to the complement of these nucleic acid molecules under stringent hybridization conditions or alternatively, under lower stringency conditions, are also encompassed by the invention, as are polypeptides encoded by these polypeptides.

Another aspect of the invention provides an isolated nucleic acid molecule comprising, or alternatively, consisting of, a polynucleotide having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding a

BGS-42 related polypeptide having an amino acid sequence as shown in the sequence listing and described in Table I; (b) a nucleotide sequence encoding a mature BGS-42 related polypeptide having the amino acid sequence as shown in the sequence listing and described in Table I; (c) a nucleotide sequence encoding a biologically active  
5 fragment of a BGS-42 related polypeptide having an amino acid sequence as shown in the sequence listing and described in Table I; (d) a nucleotide sequence encoding an antigenic fragment of a BGS-42 related polypeptide having an amino acid sequence as shown in the sequence listing and described in Table I; (e) a nucleotide sequence encoding a BGS-42 related polypeptide comprising the complete amino acid sequence  
10 encoded by a human cDNA in a cDNA plasmid contained in the ATCC Deposit and described in Table I; (f) a nucleotide sequence encoding a mature BGS-42 related polypeptide having an amino acid sequence encoded by a human cDNA in a cDNA plasmid contained in the ATCC Deposit and described in Table I; (g) a nucleotide sequence encoding a biologically active fragment of a BGS-42 related polypeptide  
15 having an amino acid sequence encoded by a human cDNA in a cDNA plasmid contained in the ATCC Deposit and described in Table I; (h) a nucleotide sequence encoding an antigenic fragment of a BGS-42 related polypeptide having an amino acid sequence encoded by a human cDNA in a cDNA plasmid contained in the ATCC deposit and described in Table I; (i) a nucleotide sequence complimentary to any of  
20 the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), or (h) above.

The present invention is also directed to nucleic acid molecules which comprise, or alternatively, consist of, a nucleotide sequence which is at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% identical to, for example, any  
25 of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), or (h), above.

The present invention encompasses polypeptide sequences which comprise, or alternatively consist of, an amino acid sequence which is at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% identical to, the following non-limited  
30 examples, the polypeptide sequence identified as SEQ ID NO:2, the polypeptide sequence encoded by a cDNA provided in the deposited clone, and/or polypeptide fragments of any of the polypeptides provided herein. Polynucleotides encoded by

these nucleic acid molecules are also encompassed by the invention. In another embodiment, the invention encompasses nucleic acid molecules which comprise, or alternatively, consist of a polynucleotide which hybridizes under stringent conditions, or alternatively, under lower stringency conditions, to a polynucleotide in (a), (b), (c),  
5 (d), (e), (f), (g), or (h), above. Polynucleotides which hybridize to the complement of these nucleic acid molecules under stringent hybridization conditions or alternatively, under lower stringency conditions, are also encompassed by the invention, as are polypeptides encoded by these polypeptides.

The present invention is also directed to polypeptides which comprise, or  
10 alternatively consist of, an amino acid sequence which is at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% identical to, for example, the polypeptide sequence shown in SEQ ID NO:2, a polypeptide sequence encoded by the nucleotide sequence in SEQ ID NO:1, a polypeptide sequence encoded by the cDNA  
15 in cDNA plasmid:Z, and/or polypeptide fragments of any of these polypeptides (e.g., those fragments described herein). Polynucleotides which hybridize to the complement of the nucleic acid molecules encoding these polypeptides under stringent hybridization conditions or alternatively, under lower stringency conditions, are also encompassed by the present invention, as are the polypeptides encoded by  
20 these polynucleotides.

By a nucleic acid having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the nucleic acid is identical to the reference sequence except that the nucleotide sequence may include up to five point mutations per each  
25 100 nucleotides of the reference nucleotide sequence encoding the polypeptide. In other words, to obtain a nucleic acid having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may  
30 be inserted into the reference sequence. The query sequence may be an entire sequence referenced in Table I, the ORF (open reading frame), or any fragment specified as described herein.

As a practical matter, whether any particular nucleic acid molecule or polypeptide is at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% identical to a nucleotide sequence of the present invention can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the CLUSTALW computer program (Thompson, J.D., et al., Nucleic Acids Research, 2(22):4673-4680, (1994)), which is based on the algorithm of Higgins, D.G., et al., Computer Applications in the Biosciences (CABIOS), 8(2):189-191, (1992). In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by converting U's to T's. However, the CLUSTALW algorithm automatically converts U's to T's when comparing RNA sequences to DNA sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a CLUSTALW alignment of DNA sequences to calculate percent identity via pairwise alignments are: Matrix=IUB, k-tuple=1, Number of Top Diagonals=5, Gap Penalty=3, Gap Open Penalty 10, Gap Extension Penalty=0.1, Scoring Method=Percent, Window Size=5 or the length of the subject nucleotide sequence, whichever is shorter. For multiple alignments, the following CLUSTALW parameters are preferred: Gap Opening Penalty=10; Gap Extension Parameter=0.05; Gap Separation Penalty Range=8; End Gap Separation Penalty=Off; % Identity for Alignment Delay=40%; Residue Specific Gaps:Off; Hydrophilic Residue Gap=Off; and Transition Weighting=0. The pairwise and multiple alignment parameters provided for CLUSTALW above represent the default parameters as provided with the AlignX software program (Vector NTI suite of programs, version 6.0).

The present invention encompasses the application of a manual correction to the percent identity results, in the instance where the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions. If only the local pairwise percent identity is required, no manual correction is needed. However, a manual correction may be applied to determine the global percent identity from a global polynucleotide alignment. Percent identity calculations based upon



global polynucleotide alignments are often preferred since they reflect the percent identity between the polynucleotide molecules as a whole (i.e., including any polynucleotide overhangs, not just overlapping regions), as opposed to, only local matching polynucleotides. Manual corrections for global percent identity determinations are required since the CLUSTALW program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the CLUSTALW sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above CLUSTALW program using the specified parameters, to arrive at a final percent identity score. This corrected score may be used for the purposes of the present invention. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the CLUSTALW alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score.

For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the CLUSTALW alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the CLUSTALW program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by CLUSTALW is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are required for the purposes of the present invention.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, or substituted with another amino acid. These alterations of the reference sequence may occur at the amino- or carboxy-terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% identical to, for instance, an amino acid sequence referenced in Table 1 (SEQ ID NO:2) or to the amino acid sequence encoded by cDNA contained in a deposited clone, can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the CLUSTALW computer program (Thompson, J.D., et al., Nucleic Acids Research, 2(22):4673-4680, (1994)), which is based on the algorithm of Higgins, D.G., et al., Computer Applications in the Biosciences (CABIOS), 8(2):189-191, (1992). In a sequence alignment the query and subject sequences are both amino acid sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a CLUSTALW alignment of DNA sequences to calculate percent identity via pairwise alignments are: Matrix=BLOSUM, k-tuple=1, Number of Top Diagonals=5, Gap Penalty=3, Gap Open Penalty 10, Gap Extension Penalty=0.1, Scoring Method=Percent, Window Size=5 or the length of the subject nucleotide sequence, whichever is shorter. For multiple alignments, the following CLUSTALW parameters are preferred: Gap Opening Penalty=10; Gap Extension Parameter=0.05; Gap Separation Penalty Range=8; End Gap Separation Penalty=Off;

% Identity for Alignment Delay=40%; Residue Specific Gaps:Off; Hydrophilic Residue Gap=Off; and Transition Weighting=0. The pairwise and multiple alignment parameters provided for CLUSTALW above represent the default parameters as provided with the AlignX software program (Vector NTI suite of programs, version 5 6.0).

The present invention encompasses the application of a manual correction to the percent identity results, in the instance where the subject sequence is shorter than the query sequence because of N- or C-terminal deletions, not because of internal deletions. If only the local pairwise percent identity is required, no manual correction is needed. However, a manual correction may be applied to determine the global percent identity from a global polypeptide alignment. Percent identity calculations based upon global polypeptide alignments are often preferred since they reflect the percent identity between the polypeptide molecules as a whole (i.e., including any polypeptide overhangs, not just overlapping regions), as opposed to, only local matching polypeptides. Manual corrections for global percent identity determinations are required since the CLUSTALW program does not account for N- and C-terminal truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the CLUSTALW sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above CLUSTALW program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what may be used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-

terminus of the subject sequence and therefore, the CLUSTALW alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C- termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the CLUSTALW program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence, which are not matched/aligned with the query. In this case the percent identity calculated by CLUSTALW is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the CLUSTALW alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are required for the purposes of the present invention.

In addition to the above method of aligning two or more polynucleotide or polypeptide sequences to arrive at a percent identity value for the aligned sequences, it may be desirable in some circumstances to use a modified version of the CLUSTALW algorithm which takes into account known structural features of the sequences to be aligned, such as for example, the SWISS-PROT designations for each sequence. The result of such a modified CLUSTALW algorithm may provide a more accurate value of the percent identity for two polynucleotide or polypeptide sequences. Support for such a modified version of CLUSTALW is provided within the CLUSTALW algorithm and would be readily appreciated to one of skill in the art of bioinformatics.

The variants may contain alterations in the coding regions, non-coding regions, or both. Especially preferred are polynucleotide variants containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. Nucleotide variants produced by silent substitutions due to the degeneracy of the genetic code are preferred. Moreover, variants in which 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination are also preferred. Polynucleotide variants can be produced

for a variety of reasons, e.g., to optimize codon expression for a particular host (change codons in the mRNA to those preferred by a bacterial host such as *E. coli*).

Naturally occurring variants are called "allelic variants" and refer to one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. (Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985).) These  
5 allelic variants can vary at either the polynucleotide and/or polypeptide level and are included in the present invention. Alternatively, non-naturally occurring variants may be produced by mutagenesis techniques or by direct synthesis.

Using known methods of protein engineering and recombinant DNA  
10 technology, variants may be generated to improve or alter the characteristics of the polypeptides of the present invention. For instance, one or more amino acids can be deleted from the N-terminus or C-terminus of the protein without substantial loss of biological function. The authors of Ron et al., *J. Biol. Chem.* 268: 2984-2988 (1993), reported variant KGF proteins having heparin binding activity even after deleting 3, 8,  
15 or 27 amino-terminal amino acid residues. Similarly, Interferon gamma exhibited up to ten times higher activity after deleting 8-10 amino acid residues from the carboxy terminus of this protein (Dobeli et al., *J. Biotechnology* 7:199-216 (1988)).

Moreover, ample evidence demonstrates that variants often retain a biological activity similar to that of the naturally occurring protein. For example, Gayle and  
20 coworkers (*J. Biol. Chem.*... 268:22105-22111 (1993)) conducted extensive mutational analysis of human cytokine IL-1a. They used random mutagenesis to generate over 3,500 individual IL-1a mutants that averaged 2.5 amino acid changes per variant over the entire length of the molecule. Multiple mutations were examined at every possible amino acid position. The investigators found that "[m]ost of the molecule could be  
25 altered with little effect on either [binding or biological activity]." In fact, only 23 unique amino acid sequences, out of more than 3,500 nucleotide sequences examined, produced a protein that significantly differed in activity from wild-type.

Furthermore, even if deleting one or more amino acids from the N-terminus or C-terminus of a polypeptide results in modification or loss of one or more biological  
30 functions, other biological activities may still be retained. For example, the ability of a deletion variant to induce and/or to bind antibodies which recognize the protein will likely be retained when less than the majority of the residues of the protein are

removed from the N-terminus or C-terminus. Whether a particular polypeptide lacking N- or C-terminal residues of a protein retains such immunogenic activities can readily be determined by routine methods described herein and otherwise known in the art.

5           Alternatively, such N-terminus or C-terminus deletions of a polypeptide of the present invention may, in fact, result in a significant increase in one or more of the biological activities of the polypeptide(s). For example, biological activity of many polypeptides are governed by the presence of regulatory domains at either one or both termini. Such regulatory domains effectively inhibit the biological activity of such  
10 polypeptides in lieu of an activation event (e.g., binding to a cognate ligand or receptor, phosphorylation, proteolytic processing, etc.). Thus, by eliminating the regulatory domain of a polypeptide, the polypeptide may effectively be rendered biologically active in the absence of an activation event.

          Thus, the invention further includes polypeptide variants that show substantial  
15 biological activity. Such variants include deletions, insertions, inversions, repeats, and substitutions selected according to general rules known in the art so as have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie et al., Science 247:1306-1310 (1990), wherein the authors indicate that there are two main strategies for studying the  
20 tolerance of an amino acid sequence to change.

          The first strategy exploits the tolerance of amino acid substitutions by natural selection during the process of evolution. By comparing amino acid sequences in different species, conserved amino acids can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions  
25 where substitutions have been tolerated by natural selection indicates that these positions are not critical for protein function. Thus, positions tolerating amino acid substitution could be modified while still maintaining biological activity of the protein.

          The second strategy uses genetic engineering to introduce amino acid changes  
30 at specific positions of a cloned gene to identify regions critical for protein function. For example, site directed mutagenesis or alanine-scanning mutagenesis (introduction of single alanine mutations at every residue in the molecule) can be used.

(Cunningham and Wells, Science 244:1081-1085 (1989).) The resulting mutant molecules can then be tested for biological activity.

As the authors state, these two strategies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at certain amino acid positions in the protein. For example, most buried (within the tertiary structure of the protein) amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved.

The invention encompasses polypeptides having a lower degree of identity but having sufficient similarity so as to perform one or more of the same functions performed by the polypeptide of the present invention. Similarity is determined by conserved amino acid substitution. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics (e.g., chemical properties). According to Cunningham et al above, such conservative substitutions are likely to be phenotypically silent. Additional guidance concerning which amino acid changes are likely to be phenotypically silent are found in Bowie et al., Science 247:1306-1310 (1990).

The invention encompasses polypeptides having a lower degree of identity but having sufficient similarity so as to perform one or more of the same functions performed by the polypeptide of the present invention. Similarity is determined by conserved amino acid substitution. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics (e.g., chemical properties). According to Cunningham et al above, such conservative substitutions are likely to be phenotypically silent. Additional guidance concerning which amino acid changes are likely to be phenotypically silent are found in Bowie et al., Science 247:1306-1310 (1990).

Tolerated conservative amino acid substitutions of the present invention involve replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl residues Ser and Thr; replacement of the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln, replacement of the basic residues Lys, Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp, and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly.

In addition, the present invention also encompasses the conservative substitutions provided in Table III below.

TABLE III

For Amino Acid	Code	Replace with any of:
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, $\beta$ -Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-1-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

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Aside from the uses described above, such amino acid substitutions may also increase protein or peptide stability. The invention encompasses amino acid substitutions that contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the protein or peptide sequence. Also included are substitutions that include amino acid residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g.,  $\beta$  or  $\gamma$  amino acids.

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Both identity and similarity can be readily calculated by reference to the following publications: Computational Molecular Biology, Lesk, A.M., ed., Oxford



University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Informatics Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M  
5 Stockton Press, New York, 1991.

In addition, the present invention also encompasses substitution of amino acids based upon the probability of an amino acid substitution resulting in conservation of function. Such probabilities are determined by aligning multiple  
10 genes with related function and assessing the relative penalty of each substitution to proper gene function. Such probabilities are often described in a matrix and are used by some algorithms (e.g., BLAST, CLUSTALW, GAP, etc.) in calculating percent similarity wherein similarity refers to the degree by which one amino acid may substitute for another amino acid without lose of function. An example of such a  
15 matrix is the PAM250 or BLOSUM62 matrix.

Aside from the canonical chemically conservative substitutions referenced above, the invention also encompasses substitutions which are typically not classified as conservative, but that may be chemically conservative under certain circumstances. Analysis of enzymatic catalysis for proteases, for example, has shown that certain  
20 amino acids within the active site of some enzymes may have highly perturbed pKa's due to the unique microenvironment of the active site. Such perturbed pKa's could enable some amino acids to substitute for other amino acids while conserving enzymatic structure and function. Examples of amino acids that are known to have amino acids with perturbed pKa's are the Glu-35 residue of Lysozyme, the Ile-16  
25 residue of Chymotrypsin, the His-159 residue of Papain, etc. The conservation of function relates to either anomalous protonation or anomalous deprotonation of such amino acids, relative to their canonical, non-perturbed pKa. The pKa perturbation may enable these amino acids to actively participate in general acid-base catalysis due to the unique ionization environment within the enzyme active site. Thus, substituting  
30 an amino acid capable of serving as either a general acid or general base within the microenvironment of an enzyme active site or cavity, as may be the case, in the same

or similar capacity as the wild-type amino acid, would effectively serve as a conservative amino substitution.

Besides conservative amino acid substitution, variants of the present invention include, but are not limited to, the following: (i) substitutions with one or more of the non-conserved amino acid residues, where the substituted amino acid residues may or may not be one encoded by the genetic code, or (ii) substitution with one or more of amino acid residues having a substituent group, or (iii) fusion of the mature polypeptide with another compound, such as a compound to increase the stability and/or solubility of the polypeptide (for example, polyethylene glycol), or (iv) fusion of the polypeptide with additional amino acids, such as, for example, an IgG Fc fusion region peptide, or leader or secretory sequence, or a sequence facilitating purification. Such variant polypeptides are deemed to be within the scope of those skilled in the art from the teachings herein.

For example, polypeptide variants containing amino acid substitutions of charged amino acids with other charged or neutral amino acids may produce proteins with improved characteristics, such as less aggregation. Aggregation of pharmaceutical formulations both reduces activity and increases clearance due to the aggregate's immunogenic activity. (Pinckard et al., Clin. Exp. Immunol. 2:331-340 (1967); Robbins et al., Diabetes 36: 838-845 (1987); Cleland et al., Crit. Rev. Therapeutic Drug Carrier Systems 10:307-377 (1993).)

Moreover, the invention further includes polypeptide variants created through the application of molecular evolution ("DNA Shuffling") methodology to the polynucleotide disclosed as SEQ ID NO:1, the sequence of the clone submitted in a deposit, and/or the cDNA encoding the polypeptide disclosed as SEQ ID NO:2. Such DNA Shuffling technology is known in the art and more particularly described elsewhere herein (e.g., WPC, Stemmer, PNAS, 91:10747, (1994)), and in the Examples provided herein).

A further embodiment of the invention relates to a polypeptide which comprises the amino acid sequence of the present invention having an amino acid sequence which contains at least one amino acid substitution, but not more than 50 amino acid substitutions, even more preferably, not more than 40 amino acid substitutions, still more preferably, not more than 30 amino acid substitutions, and

still even more preferably, not more than 20 amino acid substitutions. Of course, in order of ever-increasing preference, it is highly preferable for a peptide or polypeptide to have an amino acid sequence which comprises the amino acid sequence of the present invention, which contains at least one, but not more than 10, 9, 8, 7, 6, 5, 4, 3,  
5 2 or 1 amino acid substitutions. In specific embodiments, the number of additions, substitutions, and/or deletions in the amino acid sequence of the present invention or fragments thereof (e.g., the mature form and/or other fragments described herein), is 1-5, 5-10, 5-25, 5-50, 10-50 or 50-150, conservative amino acid substitutions are preferable.

#### 10 Polynucleotide and Polypeptide Fragments

The present invention is directed to polynucleotide fragments of the polynucleotides of the invention, in addition to polypeptides encoded therein by said polynucleotides and/or fragments.

In the present invention, a "polynucleotide fragment" refers to a short  
15 polynucleotide having a nucleic acid sequence which: is a portion of that contained in a deposited clone, or encoding the polypeptide encoded by the cDNA in a deposited clone; is a portion of that shown in SEQ ID NO:1 or the complementary strand thereto, or is a portion of a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2. The nucleotide fragments of the invention are preferably at least about 15  
20 nt, and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt, at least about 50 nt, at least about 75 nt, or at least about 150 nt in length. A fragment "at least 20 nt in length" for example, is intended to include 20 or more contiguous bases from the cDNA sequence contained in a deposited clone or the nucleotide sequence shown in SEQ ID NO:1. In  
25 this context "about" includes the particularly recited value, a value larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus, or at both termini. These nucleotide fragments have uses that include, but are not limited to, as diagnostic probes and primers as discussed herein. Of course, larger fragments (e.g., 50, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575,  
30 600, 650, 700, 750, 850, 900, 950, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000 nucleotides) are preferred.

Moreover, representative examples of polynucleotide fragments of the invention, include, for example, fragments comprising, or alternatively consisting of, a sequence from about nucleotide number 1-50, 51-100, 101-150, 151-200, 201-250, 251-300, 301-350, 351-400, 401-450, 451-500, 501-550, 551-600, 651-700, 701-750, 751-800, 800-850, 851-900, 901-950, 951-1000, 1001-1050, 1051-1100, 1101-1150, 1151-1200, 1201-1250, 1251-1300, 1301-1350, 1351-1400, 1401-1450, 1451-1500, 1501-1550, 1551-1600, 1601-1650, 1651-1700, 1701-1750, 1751-1800, 1801-1850, 1851-1900, 1901-1950, 1951-2000, or 2001 to the end of SEQ ID NO:1, or the complementary strand thereto, or the cDNA contained in a deposited clone. In this context "about" includes the particularly recited ranges, and ranges larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini. Preferably, these fragments encode a polypeptide which has biological activity. More preferably, these polynucleotides can be used as probes or primers as discussed herein. Also encompassed by the present invention are polynucleotides which hybridize to these nucleic acid molecules under stringent hybridization conditions or lower stringency conditions, as are the polypeptides encoded by these polynucleotides.

In the present invention, a "polypeptide fragment" refers to an amino acid sequence which is a portion of that contained in SEQ ID NO:2 or encoded by the cDNA contained in a deposited clone. Protein (polypeptide) fragments may be "free-standing" or comprised within a larger polypeptide of which the fragment forms a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments comprising, or alternatively consisting of, from about amino acid number 1-20, 21-40, 41-60, 61-80, 81-100, 102-120, 121-140, 141-160, or 161 to the end of the coding region. Moreover, polypeptide fragments can be about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 amino acids in length. In this context "about" includes the particularly recited ranges or values, and ranges or values larger or smaller by several (5, 4, 3, 2, or 1) amino acids, at either extreme or at both extremes. Polynucleotides encoding these polypeptides are also encompassed by the invention.

Preferred polypeptide fragments include the full-length protein. Further preferred polypeptide fragments include the full-length protein having a continuous

series of deleted residues from the amino or the carboxy terminus, or both. For example, any number of amino acids, ranging from 1-60, can be deleted from the amino terminus of the full-length polypeptide. Similarly, any number of amino acids, ranging from 1-30, can be deleted from the carboxy terminus of the full-length protein. Furthermore, any combination of the above amino and carboxy terminus deletions are preferred. Similarly, polynucleotides encoding these polypeptide fragments are also preferred.

Also preferred are polypeptide and polynucleotide fragments characterized by structural or functional domains, such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Polypeptide fragments of SEQ ID NO:2 falling within conserved domains are specifically contemplated by the present invention. Moreover, polynucleotides encoding these domains are also contemplated.

Other preferred polypeptide fragments are biologically active fragments. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of the polypeptide of the present invention. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity. Polynucleotides encoding these polypeptide fragments are also encompassed by the invention.

In a preferred embodiment, the functional activity displayed by a polypeptide encoded by a polynucleotide fragment of the invention may be one or more biological activities typically associated with the full-length polypeptide of the invention. Illustrative of these biological activities includes the fragments ability to bind to at least one of the same antibodies which bind to the full-length protein, the fragments ability to interact with at least one of the same proteins which bind to the full-length, the fragments ability to elicit at least one of the same immune responses as the full-length protein (i.e., to cause the immune system to create antibodies specific to the same epitope, etc.), the fragments ability to bind to at least one of the same polynucleotides as the full-length protein, the fragments ability to bind to a receptor of

the full-length protein, the fragments ability to bind to a ligand of the full-length protein, and the fragments ability to multimerize with the full-length protein. However, the skilled artisan would appreciate that some fragments may have biological activities which are desirable and directly inapposite to the biological activity of the full-length protein. The functional activity of polypeptides of the invention, including fragments, variants, derivatives, and analogs thereof can be determined by numerous methods available to the skilled artisan, some of which are described elsewhere herein.

The present invention encompasses polypeptides comprising, or alternatively consisting of, an epitope of the polypeptide having an amino acid sequence of SEQ ID NO:2, or an epitope of the polypeptide sequence encoded by a polynucleotide sequence contained in ATCC deposit No. Z or encoded by a polynucleotide that hybridizes to the complement of the sequence of SEQ ID NO:1 or contained in ATCC deposit No. Z under stringent hybridization conditions or lower stringency hybridization conditions as defined supra. The present invention further encompasses polynucleotide sequences encoding an epitope of a polypeptide sequence of the invention (such as, for example, the sequence disclosed in SEQ ID NO:1), polynucleotide sequences of the complementary strand of a polynucleotide sequence encoding an epitope of the invention, and polynucleotide sequences which hybridize to the complementary strand under stringent hybridization conditions or lower stringency hybridization conditions defined supra.

The term "epitopes" as used herein, refers to portions of a polypeptide having antigenic or immunogenic activity in an animal, preferably a mammal, and most preferably in a human. In a preferred embodiment, the present invention encompasses a polypeptide comprising an epitope, as well as the polynucleotide encoding this polypeptide. An "immunogenic epitope" as used herein, is defined as a portion of a protein that elicits an antibody response in an animal, as determined by any method known in the art, for example, by the methods for generating antibodies described infra. (See, for example, Geysen et al., Proc. Natl. Acad. Sci. USA 81:3998- 4002 (1983)). The term "antigenic epitope" as used herein, is defined as a portion of a protein to which an antibody can immunospecifically bind its antigen as determined by any method well known in the art, for example, by the immunoassays described

herein. Immunospecific binding excludes non-specific binding but does not necessarily exclude cross-reactivity with other antigens. Antigenic epitopes need not necessarily be immunogenic.

Fragments which function as epitopes may be produced by any conventional means. (See, e.g., Houghten, Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985),  
5 further described in U.S. Patent No. 4,631,211).

In the present invention, antigenic epitopes preferably contain a sequence of at least 4, at least 5, at least 6, at least 7, more preferably at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 20, at least 25, at  
10 least 30, at least 40, at least 50, and, most preferably, between about 15 to about 30 amino acids. Preferred polypeptides comprising immunogenic or antigenic epitopes are at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acid residues in length, or longer. Additional non-exclusive preferred antigenic epitopes include the antigenic epitopes disclosed herein, as well as portions thereof.  
15 Antigenic epitopes are useful, for example, to raise antibodies, including monoclonal antibodies, that specifically bind the epitope. Preferred antigenic epitopes include the antigenic epitopes disclosed herein, as well as any combination of two, three, four, five or more of these antigenic epitopes. Antigenic epitopes can be used as the target molecules in immunoassays. (See, for instance, Wilson et al., Cell 37:767-778 (1984);  
20 Sutcliffe et al., Science 219:660-666 (1983)).

Similarly, immunogenic epitopes can be used, for example, to induce antibodies according to methods well known in the art. (See, for instance, Sutcliffe et al., supra; Wilson et al., supra; Chow et al., Proc. Natl. Acad. Sci. USA 82:910-914; and Bittle et al., J. Gen. Virol. 66:2347-2354 (1985)). Preferred immunogenic epitopes  
25 include the immunogenic epitopes disclosed herein, as well as any combination of two, three, four, five or more of these immunogenic epitopes. The polypeptides comprising one or more immunogenic epitopes may be presented for eliciting an antibody response together with a carrier protein, such as an albumin, to an animal system (such as rabbit or mouse), or, if the polypeptide is of sufficient length (at least  
30 about 25 amino acids), the polypeptide may be presented without a carrier. However, immunogenic epitopes comprising as few as 8 to 10 amino acids have been shown to

be sufficient to raise antibodies capable of binding to, at the very least, linear epitopes in a denatured polypeptide (e.g., in Western blotting).

Epitope-bearing polypeptides of the present invention may be used to induce antibodies according to methods well known in the art including, but not limited to, in vivo immunization, in vitro immunization, and phage display methods. See, e.g.,  
5 Sutcliffe et al., supra; Wilson et al., supra, and Bittle et al., J. Gen. Virol., 66:2347-2354 (1985). If in vivo immunization is used, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus  
10 toxoid. For instance, peptides containing cysteine residues may be coupled to a carrier using a linker such as maleimidobenzoyl- N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carriers using a more general linking agent such as glutaraldehyde. Animals such as rabbits, rats and mice are immunized with either free or carrier- coupled peptides, for instance, by intraperitoneal and/or intradermal  
15 injection of emulsions containing about 100 µg of peptide or carrier protein and Freund's adjuvant or any other adjuvant known for stimulating an immune response. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of  
20 anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

As one of skill in the art will appreciate, and as discussed above, the  
25 polypeptides of the present invention comprising an immunogenic or antigenic epitope can be fused to other polypeptide sequences. For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof) resulting in chimeric polypeptides. Such fusion proteins  
30 may facilitate purification and may increase half-life in vivo. This has been shown for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of



mammalian immunoglobulins. See, e.g., EP 394,827; Traunecker et al., *Nature*, 331:84-86 (1988). Enhanced delivery of an antigen across the epithelial barrier to the immune system has been demonstrated for antigens (e.g., insulin) conjugated to an FcRn binding partner such as IgG or Fc fragments (see, e.g., PCT Publications WO 96/22024 and WO 99/04813). IgG Fusion proteins that have a disulfide-linked dimeric structure due to the IgG portion disulfide bonds have also been found to be more efficient in binding and neutralizing other molecules than monomeric polypeptides or fragments thereof alone. See, e.g., Fountoulakis et al., *J. Biochem.*, 270:3958-3964 (1995). Nucleic acids encoding the above epitopes can also be recombined with a gene of interest as an epitope tag (e.g., the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:8972- 897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix binding domain for the fusion protein. Extracts from cells infected with the recombinant vaccinia virus are loaded onto Ni<sup>2+</sup>-nitriloacetic acid-agarose column and histidine-tagged proteins can be selectively eluted with imidazole-containing buffers.

Additional fusion proteins of the invention may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to modulate the activities of polypeptides of the invention, such methods can be used to generate polypeptides with altered activity, as well as agonists and antagonists of the polypeptides. See, generally, U.S. Patent Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten et al., *Curr. Opinion Biotechnol.* 8:724-33 (1997); Harayama, *Trends Biotechnol.* 16(2):76-82 (1998); Hansson, et al., *J. Mol. Biol.* 287:265-76 (1999); and Lorenzo and Blasco, *Biotechniques* 24(2):308- 13 (1998) (each of these patents and publications are hereby incorporated by reference in its entirety). In one embodiment, alteration of polynucleotides corresponding to SEQ ID NO:1 and the polypeptides encoded by these polynucleotides may be achieved by

DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments by homologous or site-specific recombination to generate variation in the polynucleotide sequence. In another embodiment, polynucleotides of the invention, or the encoded polypeptides, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of a polynucleotide encoding a polypeptide of the invention may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

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### Antibodies

Further polypeptides of the invention relate to antibodies and T-cell antigen receptors (TCR) which immunospecifically bind a polypeptide, polypeptide fragment, or variant of SEQ ID NO:2, and/or an epitope, of the present invention (as determined by immunoassays well known in the art for assaying specific antibody-antigen binding). Antibodies of the invention include, but are not limited to, polyclonal, monoclonal, monovalent, bispecific, heteroconjugate, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. Moreover, the term "antibody" (Ab) or "monoclonal antibody" (Mab) is meant to include intact molecules, as well as, antibody fragments (such as, for example, Fab and F(ab')<sub>2</sub> fragments) which are capable of specifically binding to protein. Fab and F(ab')<sub>2</sub> fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation of the animal or plant, and may have less non-specific tissue binding than an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)). Thus, these fragments are preferred, as well as the products of a FAB or other immunoglobulin

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expression library. Moreover, antibodies of the present invention include chimeric, single chain, and humanized antibodies.

Most preferably the antibodies are human antigen-binding antibody fragments of the present invention and include, but are not limited to, Fab, Fab' and F(ab')<sub>2</sub>, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and CH3 domains. The antibodies of the invention may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine (e.g., mouse and rat), donkey, sheep rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulin and that do not express endogenous immunoglobulins, as described infra and, for example in, U.S. Patent No. 5,939,598 by Kucherlapati et al.

The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of a polypeptide of the present invention or may be specific for both a polypeptide of the present invention as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material. See, e.g., PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, et al., J. Immunol. 147:60-69 (1991); U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny et al., J. Immunol. 148:1547-1553 (1992).

Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide of the present invention which they recognize or specifically bind. The epitope(s) or polypeptide portion(s) may be specified as described herein, e.g., by N-terminal and C-terminal positions, by size in contiguous amino acid residues, or listed in the Tables and Figures. Antibodies which specifically bind any epitope or polypeptide of the present invention may also be

excluded. Therefore, the present invention includes antibodies that specifically bind polypeptides of the present invention, and allows for the exclusion of the same.

Antibodies of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies that do not bind any other analog, ortholog, or homologue of a polypeptide of the present invention are included. Antibodies that  
5 bind polypeptides with at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, and at least 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. In specific  
10 embodiments, antibodies of the present invention cross-react with murine, rat and/or rabbit homologues of human proteins and the corresponding epitopes thereof. Antibodies that do not bind polypeptides with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art  
15 and described herein) to a polypeptide of the present invention are also included in the present invention. In a specific embodiment, the above-described cross-reactivity is with respect to any single specific antigenic or immunogenic polypeptide, or combination(s) of 2, 3, 4, 5, or more of the specific antigenic and/or immunogenic polypeptides disclosed herein. Further included in the present invention are antibodies  
20 which bind polypeptides encoded by polynucleotides which hybridize to a polynucleotide of the present invention under stringent hybridization conditions (as described herein). Antibodies of the present invention may also be described or specified in terms of their binding affinity to a polypeptide of the invention. Preferred binding affinities include those with a dissociation constant or  $K_d$  less than  $5 \times 10^{-2}$   
25 M,  $10^{-2}$  M,  $5 \times 10^{-3}$  M,  $10^{-3}$  M,  $5 \times 10^{-4}$  M,  $10^{-4}$  M,  $5 \times 10^{-5}$  M,  $10^{-5}$  M,  $5 \times 10^{-6}$  M,  $10^{-6}$  M,  $5 \times 10^{-7}$  M,  $10^{-7}$  M,  $5 \times 10^{-8}$  M,  $10^{-8}$  M,  $5 \times 10^{-9}$  M,  $10^{-9}$  M,  $5 \times 10^{-10}$  M,  $10^{-10}$  M,  $5 \times 10^{-11}$  M,  $10^{-11}$  M,  $5 \times 10^{-12}$  M,  $10^{-12}$  M,  $5 \times 10^{-13}$  M,  $10^{-13}$  M,  $5 \times 10^{-14}$  M,  $10^{-14}$  M,  $5 \times 10^{-15}$  M, or  $10^{-15}$  M.

The invention also provides antibodies that competitively inhibit binding of an  
30 antibody to an epitope of the invention as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the antibody competitively inhibits binding to the

epitope by at least 95%, at least 90%, at least 85 %, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50%.

Antibodies of the present invention may act as agonists or antagonists of the polypeptides of the present invention. For example, the present invention includes  
5 antibodies which disrupt the receptor/ligand interactions with the polypeptides of the invention either partially or fully. Preferably, antibodies of the present invention bind an antigenic epitope disclosed herein, or a portion thereof. The invention features both receptor-specific antibodies and ligand-specific antibodies. The invention also features receptor-specific antibodies which do not prevent ligand binding but prevent  
10 receptor activation. Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. For example, receptor activation can be determined by detecting the phosphorylation (e.g., tyrosine or serine/threonine) of the receptor or its substrate by immunoprecipitation followed by western blot analysis (for example, as described supra). In specific embodiments,  
15 antibodies are provided that inhibit ligand activity or receptor activity by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50% of the activity in absence of the antibody.

The invention also features receptor-specific antibodies which both prevent ligand binding and receptor activation as well as antibodies that recognize the  
20 receptor-ligand complex, and, preferably, do not specifically recognize the unbound receptor or the unbound ligand. Likewise, included in the invention are neutralizing antibodies which bind the ligand and prevent binding of the ligand to the receptor, as well as antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. Further included in the invention  
25 are antibodies which activate the receptor. These antibodies may act as receptor agonists, i.e., potentiate or activate either all or a subset of the biological activities of the ligand-mediated receptor activation, for example, by inducing dimerization of the receptor. The antibodies may be specified as agonists, antagonists or inverse agonists for biological activities comprising the specific biological activities of the peptides of  
30 the invention disclosed herein. The above antibody agonists can be made using methods known in the art. See, e.g., PCT publication WO 96/40281; U.S. Patent No. 5,811,097; Deng et al., Blood 92(6):1981-1988 (1998); Chen et al., Cancer Res.

58(16):3668-3678 (1998); Harrop et al., J. Immunol. 161(4):1786-1794 (1998); Zhu et al., Cancer Res. 58(15):3209-3214 (1998); Yoon et al., J. Immunol. 160(7):3170-3179 (1998); Prat et al., J. Cell. Sci. 111(Pt2):237-247 (1998); Pitard et al., J. Immunol. Methods 205(2):177-190 (1997); Liautard et al., Cytokine 9(4):233-241 (1997);  
5 Carlson et al., J. Biol. Chem.. 272(17):11295-11301 (1997); Taryman et al., Neuron 14(4):755-762 (1995); Muller et al., Structure 6(9):1153-1167 (1998); Bartunek et al., Cytokine 8(1):14-20 (1996) (which are all incorporated by reference herein in their entireties).

Antibodies of the present invention may be used, for example, but not limited  
10 to, to purify, detect, and target the polypeptides of the present invention, including both in vitro and in vivo diagnostic and therapeutic methods. For example, the antibodies have use in immunoassays for qualitatively and quantitatively measuring levels of the polypeptides of the present invention in biological samples. See, e.g., Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory  
15 Press, 2nd ed. 1988) (incorporated by reference herein in its entirety).

As discussed in more detail below, the antibodies of the present invention may be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to  
20 polypeptides or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, radionucleotides, or toxins. See, e.g., PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 396,387.

25 The antibodies of the invention include derivatives that are modified, i.e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation,  
30 phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including,

but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

5       The antibodies of the present invention may be generated by any suitable method known in the art.

      The antibodies of the present invention may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan (Harlow, et al., *Antibodies: A Laboratory Manual*, (Cold spring Harbor Laboratory Press, 2<sup>nd</sup> ed. (1988); and *Current Protocols*, Chapter 2; which are hereby incorporated herein by  
10       reference in its entirety). In a preferred method, a preparation of the BGS-42 protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity. For example, a polypeptide of the invention can be administered to various host animals including, but not limited to, rabbits, mice, rats,  
15       etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. The administration of the polypeptides of the present invention may entail one or more injections of an immunizing agent and, if desired, an adjuvant. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete),  
20       mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art. For the purposes of the invention, "immunizing agent" may be  
25       defined as a polypeptide of the invention, including fragments, variants, and/or derivatives thereof, in addition to fusions with heterologous polypeptides and other forms of the polypeptides described herein.

      Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections, though they may also  
30       be given intramuscularly, and/or through IV). The immunizing agent may include polypeptides of the present invention or a fusion protein or variants thereof. Depending upon the nature of the polypeptides (i.e., percent hydrophobicity, percent

hydrophilicity, stability, net charge, isoelectric point etc.), it may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Such conjugation includes either chemical conjugation by derivitizing active chemical functional groups to both the polypeptide of the present invention and the immunogenic protein such that a covalent bond is formed, or through fusion-protein based methodology, or other methods known to the skilled artisan. Examples of such immunogenic proteins include, but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Additional examples of adjuvants which may be employed includes the MPL-TDM adjuvant (monophosphoryl lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

The antibodies of the present invention may comprise monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975) and U.S. Pat. No. 4,376,110, by Harlow, et al., *Antibodies: A Laboratory Manual*, (Cold spring Harbor Laboratory Press, 2<sup>nd</sup> ed. (1988), by Hammerling, et al., *Monoclonal Antibodies and T-Cell Hybridomas* (Elsevier, N.Y., pp. 563-681 (1981); Köhler et al., *Eur. J. Immunol.* 6:511 (1976); Köhler et al., *Eur. J. Immunol.* 6:292 (1976), or other methods known to the artisan. Other examples of methods which may be employed for producing monoclonal antibodies includes, but are not limited to, the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* 4:72; Cole et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this



invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In a hybridoma method, a mouse, a humanized mouse, a mouse with a human immune system, hamster, or other appropriate host animal, is typically immunized  
5 with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized in vitro.

The immunizing agent will typically include polypeptides of the present invention or a fusion protein thereof. Preferably, the immunizing agent consists of an  
10 BGS-42 polypeptide or, more preferably, with a BGS-42 polypeptide-expressing cell. Such cells may be cultured in any suitable tissue culture medium; however, it is preferable to culture cells in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56 degrees C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100  
15 ug/ml of streptomycin. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice,  
20 Academic Press, (1986), pp. 59-103). Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if  
25 the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable  
30 high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute

Cell Distribution Center, San Diego, California and the American Type Culture Collection, Manassas, Virginia. More preferred are the parent myeloma cell line (SP2O) as provided by the ATCC. As inferred throughout the specification, human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63).

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against the polypeptides of the present invention. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbant assay (ELISA). Such techniques are known in the art and within the skill of the artisan. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollart, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, supra, and/or according to Wands et al. (Gastroenterology 80:225-232 (1981))). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640. Alternatively, the hybridoma cells may be grown in vivo as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-sepharose, hydroxyapatite chromatography, gel exclusion chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The skilled artisan would acknowledge that a variety of methods exist in the art for the production of monoclonal antibodies and thus, the invention is not limited to their sole production in hybridomas. For example, the monoclonal antibodies may be made by recombinant DNA methods, such as those described in US patent No. 4, 816, 567. In this context, the term "monoclonal antibody" refers to an antibody

derived from a single eukaryotic, phage, or prokaryotic clone. The DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies, or such chains from human, humanized, or other sources). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transformed into host cells such as Simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (US Patent No. 4, 816, 567; Morrison et al, supra) or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988);

Hammerling, et al., in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art and are discussed in detail in the Examples described herein. In a non-limiting example, mice can be immunized with a polypeptide of the invention or a cell expressing such peptide. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of the invention. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

Accordingly, the present invention provides methods of generating monoclonal antibodies as well as antibodies produced by the method comprising culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with an antigen of the invention with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of the invention.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')<sub>2</sub> fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')<sub>2</sub> fragments). F(ab')<sub>2</sub> fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

For example, the antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., *J. Immunol. Methods* 182:41-50 (1995); Ames et al., *J. Immunol. Methods* 184:177-186 (1995); Kettleborough et al., *Eur. J. Immunol.* 24:952-958 (1994); Persic et al., *Gene* 187 9-18 (1997); Burton et al., *Advances in Immunology* 57:191-280 (1994); PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')<sub>2</sub> fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al., *BioTechniques* 12(6):864-869 (1992); and Sawai et al., *AJRI* 34:26-34 (1995); and Better et al., *Science* 240:1041-1043 (1988) (said references incorporated by reference in their entireties). Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S.

Patents 4,946,778 and 5,258,498; Huston et al., *Methods in Enzymology* 203:46-88 (1991); Shu et al., *PNAS* 90:7995-7999 (1993); and Skerra et al., *Science* 240:1038-1040 (1988).

For some uses, including in vivo use of antibodies in humans and in vitro  
5 detection assays, it may be preferable to use chimeric, humanized, or human  
antibodies. A chimeric antibody is a molecule in which different portions of the  
antibody are derived from different animal species, such as antibodies having a  
variable region derived from a murine monoclonal antibody and a human  
immunoglobulin constant region. Methods for producing chimeric antibodies are  
10 known in the art. See e.g., Morrison, *Science* 229:1202 (1985); Oi et al.,  
*BioTechniques* 4:214 (1986); Gillies et al., (1989) *J. Immunol. Methods* 125:191-202;  
Cabilly et al., Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et  
al., WO 8601533; Robinson et al., WO 8702671; Boulianne et al., *Nature* 312:643  
(1984); Neuberger et al., *Nature* 314:268 (1985); U.S. Patent Nos. 5,807,715;  
15 4,816,567; and 4,816,397, which are incorporated herein by reference in their entirety.  
Humanized antibodies are antibody molecules from non-human species antibody that  
binds the desired antigen having one or more complementarity determining regions  
(CDRs) from the non-human species and a framework regions from a human  
immunoglobulin molecule. Often, framework residues in the human framework  
20 regions will be substituted with the corresponding residue from the CDR donor  
antibody to alter, preferably improve, antigen binding. These framework substitutions  
are identified by methods well known in the art, e.g., by modeling of the interactions  
of the CDR and framework residues to identify framework residues important for  
antigen binding and sequence comparison to identify unusual framework residues at  
25 particular positions. (See, e.g., Queen et al., U.S. Patent No. 5,585,089; Riechmann et  
al., *Nature* 332:323 (1988), which are incorporated herein by reference in their  
entireties.) Antibodies can be humanized using a variety of techniques known in the  
art including, for example, CDR-grafting (EP 239,400; PCT publication WO  
91/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or  
30 resurfacing (EP 592,106; EP 519,596; Padlan, *Molecular Immunology* 28(4/5):489-  
498 (1991); Studnicka et al., *Protein Engineering* 7(6):805-814 (1994); Roguska. et  
al., *PNAS* 91:969-973 (1994)), and chain shuffling (U.S. Patent No. 5,565,332).

Generally, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the methods of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (US Patent No. 4, 816, 567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possible some FR residues are substituted from analogous sites in rodent antibodies.

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988)l and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992).

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety. The techniques of cole et al., and Boerder et al., are also available for the preparation of human monoclonal antibodies (cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Riss, (1985); and Boerner et al., *J. Immunol.*, 147(1):86-95, (1991)).

Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, *Int. Rev. Immunol.* 13:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, CA), Genpharm (San Jose, CA), and Medarex, Inc. (Princeton, NJ) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Similarly, human antibodies can be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous



immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and creation of an antibody repertoire. This approach is described, for example, in US patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,106, and in the following scientific publications: Marks et al., *Biotechnol.*, 10:779-783 (1992); Lonberg et al., *Nature* 368:856-859 (1994); Fishwild et al., *Nature Biotechnol.*, 14:845-51 (1996); Neuberger, *Nature Biotechnol.*, 14:826 (1996); Lonberg and Huszer, *Intern. Rev. Immunol.*, 13:65-93 (1995).

10 Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al., *Bio/technology* 12:899-903 (1988)).

15 Further, antibodies to the polypeptides of the invention can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" polypeptides of the invention using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, *FASEB J.* 7(5):437-444; (1989) and Nissinoff, *J. Immunol.* 147(8):2429-2438 (1991)). For example, antibodies which bind to and competitively inhibit polypeptide multimerization and/or binding of a polypeptide of the invention to a ligand can be used to generate anti-idiotypes that "mimic" the polypeptide multimerization and/or binding domain and, as a consequence, bind to and neutralize polypeptide and/or its ligand. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize polypeptide ligand. For example, such anti-idiotypic antibodies can be used to bind a polypeptide of the invention and/or to bind its ligands/receptors, and thereby block its biological activity.

20 Such anti-idiotypic antibodies capable of binding to the BGS-42 polypeptide can be produced in a two-step procedure. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody that binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are

30

screened to identify clones that produce an antibody whose ability to bind to the protein-specific antibody can be blocked by the polypeptide. Such antibodies comprise anti-idiotypic antibodies to the protein-specific antibody and can be used to immunize an animal to induce formation of further protein-specific antibodies.

5           The antibodies of the present invention may be bispecific antibodies. Bispecific antibodies are monoclonal, Preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present invention, one of the binding specificities may be directed towards a polypeptide of the present invention, the other may be for any other antigen, and preferably for a cell-surface  
10 protein, receptor, receptor subunit, tissue-specific antigen, virally derived protein, virally encoded envelope protein, bacterially derived protein, or bacterial surface protein, etc.

          Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of  
15 two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, *Nature*, 305:537-539 (1983). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct  
20 molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., *EMBO J.*, 10:3655-3659 (1991).

          Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences.  
25 The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain,  
30 are inserted into separate expression vectors, and are co-transformed into a suitable host organism. For further details of generating bispecific antibodies see, for example Suresh et al., *Meth. In Enzym.*, 121:210 (1986).

Heteroconjugate antibodies are also contemplated by the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4, 676, 980), and for the treatment of HIV infection  
5 (WO 91/00360; WO 92/20373; and EP03089). It is contemplated that the antibodies may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioester bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-  
10 mercaptobutyrimidate and those disclosed, for example, in US Patent No. 4,676,980.

#### Polynucleotides Encoding Antibodies

The invention further provides polynucleotides comprising a nucleotide sequence encoding an antibody of the invention and fragments thereof. The invention also encompasses polynucleotides that hybridize under stringent or lower stringency  
15 hybridization conditions, e.g., as defined supra, to polynucleotides that encode an antibody, preferably, that specifically binds to a polypeptide of the invention, preferably, an antibody that binds to a polypeptide having the amino acid sequence of SEQ ID NO:2.

The polynucleotides may be obtained, and the nucleotide sequence of the  
20 polynucleotides determined, by any method known in the art. For example, if the nucleotide sequence of the antibody is known, a polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides (e.g., as described in Kutmeier et al., BioTechniques 17:242 (1994)), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the  
25 antibody, annealing and ligating of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, a polynucleotide encoding an antibody may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a particular antibody is not available, but the sequence of the antibody molecule is  
30 known, a nucleic acid encoding the immunoglobulin may be chemically synthesized or obtained from a suitable source (e.g., an antibody cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from, any tissue or

cells expressing the antibody, such as hybridoma cells selected to express an antibody of the invention) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, e.g., a cDNA clone from a cDNA library that  
5 encodes the antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

Once the nucleotide sequence and corresponding amino acid sequence of the antibody is determined, the nucleotide sequence of the antibody may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, e.g., recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for  
10 example, the techniques described in Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel et al., eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties ), to  
15 generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

In a specific embodiment, the amino acid sequence of the heavy and/or light chain variable domains may be inspected to identify the sequences of the complementarity determining regions (CDRs) by methods that are well known in the art, e.g., by comparison to known amino acid sequences of other heavy and light chain  
20 variable regions to determine the regions of sequence hypervariability. Using routine recombinant DNA techniques, one or more of the CDRs may be inserted within framework regions, e.g., into human framework regions to humanize a non-human antibody, as described supra. The framework regions may be naturally occurring or  
25 consensus framework regions, and preferably human framework regions (see, e.g., Chothia et al., J. Mol. Biol. 278: 457-479 (1998) for a listing of human framework regions). Preferably, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds a polypeptide of the invention. Preferably, as discussed supra, one or more amino acid  
30 substitutions may be made within the framework regions, and, preferably, the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more

variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present invention and within the skill of the art.

5           In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., Proc. Natl. Acad. Sci. 81:851-855 (1984); Neuberger et al., Nature 312:604-608 (1984); Takeda et al., Nature 314:452-454 (1985)) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used.

10       As described supra, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region, e.g., humanized antibodies.

          Alternatively, techniques described for the production of single chain

15       antibodies (U.S. Patent No. 4,946,778; Bird, Science 242:423- 42 (1988); Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-5883 (1988); and Ward et al., Nature 334:544-54 (1989)) can be adapted to produce single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the

20       assembly of functional Fv fragments in E. coli may also be used (Skerra et al., Science 242:1038- 1041 (1988)).

          More preferably, a clone encoding an antibody of the present invention may be obtained according to the method described in the Example section herein.

#### Methods of Producing Antibodies

25       The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

          Recombinant expression of an antibody of the invention, or fragment, derivative or analog thereof, (e.g., a heavy or light chain of an antibody of the

30       invention or a single chain antibody of the invention), requires construction of an expression vector containing a polynucleotide that encodes the antibody. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an

antibody, or portion thereof (preferably containing the heavy or light chain variable domain), of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing  
5 a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic  
10 recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an antibody molecule of the invention, or a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., PCT Publication WO 86/05807; PCT  
15 Publication WO 89/01036; and U.S. Patent No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an  
20 antibody of the invention. Thus, the invention includes host cells containing a polynucleotide encoding an antibody of the invention, or a heavy or light chain thereof, or a single chain antibody of the invention, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell  
25 for expression of the entire immunoglobulin molecule, as detailed below.

A variety of host-expression vector systems may be utilized to express the antibody molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the  
30 appropriate nucleotide coding sequences, express an antibody molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid

DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing antibody coding sequences; 5 plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing antibody coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the 10 genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule, are used for the expression of a recombinant antibody molecule. For example, mammalian 15 cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al., *Gene* 45:101 (1986); Cockett et al., *Bio/Technology* 8:2 (1990)).

In bacterial systems, a number of expression vectors may be advantageously 20 selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression 25 vector pUR278 (Ruther et al., *EMBO J.* 2:1791 (1983)), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, *Nucleic Acids Res.* 13:3101-3109 (1985); Van Heeke & Schuster, *J. Biol. Chem.* 24:5503-5509 (1989)); and the like. pGEX vectors may also be used to express foreign 30 polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione-agarose beads followed by elution in the presence

of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

5 In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

10 In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region  
15 E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts. (e.g., see Logan & Shenk, *Proc. Natl. Acad. Sci. USA* 81:355-359 (1984)). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must  
20 be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al.,  
25 *Methods in Enzymol.* 153:51-544 (1987)).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein.  
30 Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and



processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, 293, 3T3, WI38, and in particular, breast cancer cell lines such as, for example, BT483, Hs578T, HTB2, BT20 and T47D, and normal mammary gland cell line such as, for example, CRL7030 and Hs578Bst.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the antibody molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibody molecule. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., Cell 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci. USA 48:202 (1992)), and adenine phosphoribosyltransferase (Lowy et al., Cell 22:817 (1980)) genes can be employed in tk-, hgp<sup>rt</sup>- or ap<sup>rt</sup>- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., Natl. Acad. Sci. USA 77:357 (1980); O'Hare et al., Proc. Natl. Acad. Sci. USA 78:1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci. USA 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G-418 Clinical Pharmacy 12:488-505; Wu and Wu, Biotherapy 3:87-95 (1991);

Tolstoshev, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596 (1993); Mulligan, *Science* 260:926-932 (1993); and Morgan and Anderson, *Ann. Rev. Biochem.* 62:191-217 (1993); May, 1993, *TIB TECH* 11(5):155-215); and hygromycin, which confers resistance to hygromycin (Santerre et al., *Gene* 30:147 (1984)). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, NY (1993); Kriegler, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli et al. (eds), *Current Protocols in Human Genetics*, John Wiley & Sons, NY (1994); Colberre-Garapin et al., *J. Mol. Biol.* 150:1 (1981), which are incorporated by reference herein in their entireties.

The expression levels of an antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, *The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning*, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse et al., *Mol. Cell. Biol.* 3:257 (1983)).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, *Nature* 322:52 (1986); Kohler, *Proc. Natl. Acad. Sci. USA* 77:2197 (1980)). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once an antibody molecule of the invention has been produced by an animal, chemically synthesized, or recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for

example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In addition, the antibodies of the present invention or  
5 fragments thereof can be fused to heterologous polypeptide sequences described herein or otherwise known in the art, to facilitate purification.

The present invention encompasses antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to a polypeptide (or portion thereof, preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90  
10 or 100 amino acids of the polypeptide) of the present invention to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. The antibodies may be specific for antigens other than polypeptides (or portion thereof, preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids of the polypeptide) of the present invention. For example, antibodies may be  
15 used to target the polypeptides of the present invention to particular cell types, either in vitro or in vivo, by fusing or conjugating the polypeptides of the present invention to antibodies specific for particular cell surface receptors. Antibodies fused or conjugated to the polypeptides of the present invention may also be used in in vitro immunoassays and purification methods using methods known in the art. See e.g.,  
20 Harbor et al., supra, and PCT publication WO 93/21232; EP 439,095; Naramura et al., Immunol. Lett. 39:91-99 (1994); U.S. Patent 5,474,981; Gillies et al., PNAS 89:1428-1432 (1992); Fell et al., J. Immunol. 146:2446-2452(1991), which are incorporated by reference in their entirety.

The present invention further includes compositions comprising the  
25 polypeptides of the present invention fused or conjugated to antibody domains other than the variable regions. For example, the polypeptides of the present invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a polypeptide of the present invention may comprise the constant region, hinge region, CH1 domain, CH2 domain, and CH3 domain or any  
30 combination of whole domains or portions thereof. The polypeptides may also be fused or conjugated to the above antibody portions to form multimers. For example, Fc portions fused to the polypeptides of the present invention can form dimers

through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the polypeptides to portions of IgA and IgM. Methods for fusing or conjugating the polypeptides of the present invention to antibody portions are known in the art. See, e.g., U.S. Patent Nos. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851; 5,112,946; EP 307,434; EP 367,166; PCT publications WO 96/04388; WO 91/06570; Ashkenazi et al., Proc. Natl. Acad. Sci. USA 88:10535-10539 (1991); Zheng et al., J. Immunol. 154:5590-5600 (1995); and Vil et al., Proc. Natl. Acad. Sci. USA 89:11337- 11341(1992) (said references incorporated by reference in their entireties).

10           As discussed, supra, the polypeptides corresponding to a polypeptide, polypeptide fragment, or a variant of SEQ ID NO:2 may be fused or conjugated to the above antibody portions to increase the in vivo half life of the polypeptides or for use in immunoassays using methods known in the art. Further, the polypeptides corresponding to SEQ ID NO:2 may be fused or conjugated to the above antibody  
15           portions to facilitate purification. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP 394,827; Traunecker et al., Nature 331:84-86 (1988). The polypeptides of the present invention fused or conjugated to an antibody having  
20           disulfide- linked dimeric structures (due to the IgG) may also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis et al., J. Biochem. 270:3958-3964 (1995)). In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP A  
25           232,262). Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to  
30           identify antagonists of hIL-5. (See, Bennett et al., J. Molecular Recognition 8:52-58 (1995); Johanson et al., J. Biol. Chem.. 270:9459-9471 (1995).

Moreover, the antibodies or fragments thereof the present invention can be fused to marker sequences, such as a peptide to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz et al., Proc. Natl. Acad. Sci. USA 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., Cell 37:767 (1984)) and the "flag" tag.

The present invention further encompasses antibodies or fragments thereof conjugated to a diagnostic or therapeutic agent. The antibodies can be used diagnostically to, for example, monitor the development or progression of a tumor as part of a clinical testing procedure to, e.g., determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. The detectable substance may be coupled or conjugated either directly to the antibody (or fragment thereof) or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. See, for example, U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{111}\text{In}$  or  $^{99}\text{Tc}$ .

Further, an antibody or fragment thereof may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters such as, for example,  $^{213}\text{Bi}$ . A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include

5 paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologues thereof. Therapeutic agents include, but are

10 not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin

15 (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response, the therapeutic agent or drug moiety is not to be construed as limited to

20 classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor,  $\alpha$ -interferon,  $\beta$ -interferon, nerve tubulin tyrosine ligase protein, platelet derived tubulin tyrosine ligase protein, tissue plasminogen

25 activator, an apoptotic agent, e.g., TNF- $\alpha$ , TNF- $\beta$ , AIM I (See, International Publication No. WO 97/33899), AIM II (See, International Publication No. WO 97/34911), Fas Ligand (Takahashi et al., Int. Immunol., 6:1567-1574 (1994)), VEGI (See, International Publication No. WO 99/23105), a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, biological response modifiers

30 such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-

CSF"), granulocyte colony stimulating factor ("G-CSF"), or other tubulin tyrosine ligase proteins.

Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports  
5 include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results,  
10 And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.* 62:119-58 (1982).  
15

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980, which is incorporated herein by reference in its entirety.  
20

An antibody, with or without a therapeutic moiety conjugated to it, administered alone or in combination with cytotoxic factor(s) and/or cytokine(s) can  
25 be used as a therapeutic.

The present invention also encompasses the creation of synthetic antibodies directed against the polypeptides of the present invention. One example of synthetic antibodies is described in Radrizzani, M., et al., *Medicina*, (Aires), 59(6):753-8, (1999)). Recently, a new class of synthetic antibodies has been described and are referred to as molecularly imprinted polymers (MIPs) (Semorex, Inc.). Antibodies,  
30 peptides, and enzymes are often used as molecular recognition elements in chemical and biological sensors. However, their lack of stability and signal transduction

mechanisms limits their use as sensing devices. Molecularly imprinted polymers (MIPs) are capable of mimicking the function of biological receptors but with less stability constraints. Such polymers provide high sensitivity and selectivity while maintaining excellent thermal and mechanical stability. MIPs have the ability to bind to small molecules and to target molecules such as organics and proteins' with equal or greater potency than that of natural antibodies. These "super" MIPs have higher affinities for their target and thus require lower concentrations for efficacious binding.

During synthesis, the MIPs are imprinted so as to have complementary size, shape, charge and functional groups of the selected target by using the target molecule itself (such as a polypeptide, antibody, etc.), or a substance having a very similar structure, as its "print" or "template." MIPs can be derivatized with the same reagents afforded to antibodies. For example, fluorescent 'super' MIPs can be coated onto beads or wells for use in highly sensitive separations or assays, or for use in high throughput screening of proteins.

Moreover, MIPs based upon the structure of the polypeptide(s) of the present invention may be useful in screening for compounds that bind to the polypeptide(s) of the invention. Such a MIP would serve the role of a synthetic "receptor" by mimicking the native architecture of the polypeptide. In fact, the ability of a MIP to serve the role of a synthetic receptor has already been demonstrated for the estrogen receptor (Ye, L., Yu, Y., Mosbach, K, *Analyst.*, 126(6):760-5, (2001); Dickert, F, L., Hayden, O., Halikias, K, P, *Analyst.*, 126(6):766-71, (2001)). A synthetic receptor may either be mimicked in its entirety (e.g., as the entire protein), or mimicked as a series of short peptides corresponding to the protein (Rachkov, A., Minoura, N, *Biochim, Biophys, Acta.*, 1544(1-2):255-66, (2001)). Such a synthetic receptor MIPs may be employed in any one or more of the screening methods described elsewhere herein.

MIPs have also been shown to be useful in "sensing" the presence of its mimicked molecule (Cheng, Z., Wang, E., Yang, X, *Biosens, Bioelectron.*, 16(3):179-85, (2001) ; Jenkins, A, L., Yin, R., Jensen, J. L, *Analyst.*, 126(6):798-802, (2001) ; Jenkins, A, L., Yin, R., Jensen, J. L, *Analyst.*, 126(6):798-802, (2001)). For example, a MIP designed using a polypeptide of the present invention may be used in assays designed to identify, and potentially quantitate, the level of said polypeptide in a



sample. Such a MIP may be used as a substitute for any component described in the assays, or kits, provided herein (e.g., ELISA, etc.).

A number of methods may be employed to create MIPs to a specific receptor, ligand, polypeptide, peptide, organic molecule. Several preferred methods are described by Esteban et al in J. Anal, Chem., 370(7):795-802, (2001), which is hereby  
5 incorporated herein by reference in its entirety in addition to any references cited therein. Additional methods are known in the art and are encompassed by the present invention, such as for example, Hart, B, R., Shea, K, J. J. Am. Chem, Soc., 123(9):2072-3, (2001); and Quaglia, M., Chenon, K., Hall, A, J., De, Lorenzi, E.,  
10 Sellergren, B, J. Am. Chem, Soc., 123(10):2146-54, (2001); which are hereby incorporated by reference in their entirety herein.

#### Uses for Antibodies directed against polypeptides of the invention

The antibodies of the present invention have various utilities. For example, such antibodies may be used in diagnostic assays to detect the presence or  
15 quantification of the polypeptides of the invention in a sample. Such a diagnostic assay may be comprised of at least two steps. The first, subjecting a sample with the antibody, wherein the sample is a tissue (e.g., human, animal, etc.), biological fluid (e.g., blood, urine, sputum, semen, amniotic fluid, saliva, etc.), biological extract (e.g., tissue or cellular homogenate, etc.), a protein microchip (e.g., See Arenkov P, et al.,  
20 Anal Biochem., 278(2):123-131 (2000)), or a chromatography column, etc. And a second step involving the quantification of antibody bound to the substrate. Alternatively, the method may additionally involve a first step of attaching the antibody, either covalently, electrostatically, or reversibly, to a solid support, and a second step of subjecting the bound antibody to the sample, as defined above and  
25 elsewhere herein.

Various diagnostic assay techniques are known in the art, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogenous phases (Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc., (1987), pp147-158). The  
30 antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as

2H, <sup>14</sup>C, <sup>32</sup>P, or <sup>125</sup>I, a florescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase, green fluorescent protein, or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety  
5 may be employed, including those methods described by Hunter et al., *Nature*, 144:945 (1962); Dafvid et al., *Biochem.*, 13:1014 (1974); Pain et al., *J. Immunol. Metho.*, 40:219(1981); and Nygren, *J. Histochem. And Cytochem.*, 30:407 (1982).

Antibodies directed against the polypeptides of the present invention are useful for the affinity purification of such polypeptides from recombinant cell culture  
10 or natural sources. In this process, the antibodies against a particular polypeptide are immobilized on a suitable support, such as a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the polypeptides to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the  
15 sample except for the desired polypeptides, which are bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the desired polypeptide from the antibody.

#### Immunophenotyping

The antibodies of the invention may be utilized for immunophenotyping of  
20 cell lines and biological samples. The translation product of the gene of the present invention may be useful as a cell specific marker, or more specifically as a cellular marker that is differentially expressed at various stages of differentiation and/or maturation of particular cell types. Monoclonal antibodies directed against a specific epitope, or combination of epitopes, will allow for the screening of cellular  
25 populations expressing the marker. Various techniques can be utilized using monoclonal antibodies to screen for cellular populations expressing the marker(s), and include magnetic separation using antibody-coated magnetic beads, "panning" with antibody attached to a solid matrix (i.e., plate), and flow cytometry (See, e.g., U.S. Patent 5,985,660; and Morrison et al., *Cell*, 96:737-49 (1999)).

30 These techniques allow for the screening of particular populations of cells, such as might be found with hematological malignancies (i.e. minimal residual disease (MRD) in acute leukemic patients) and "non-self" cells in transplantations to

prevent Graft-versus-Host Disease (GVHD). Alternatively, these techniques allow for the screening of hematopoietic stem and progenitor cells capable of undergoing proliferation and/or differentiation, as might be found in human umbilical cord blood.

#### Assays For Antibody Binding

5           The antibodies of the invention may be assayed for immunospecific binding by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions,  
10   gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference  
15   herein in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

          Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X- 100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1%  
20   Trasylol) supplemented with protein phosphatase and/or protease inhibitors (e.g., EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell lysate, incubating for a period of time (e.g., 1-4 hours) at 4° C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4° C, washing the beads in lysis buffer and resuspending the beads in  
25   SDS/sample buffer. The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, e.g., western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background (e.g., pre-clearing the cell lysate with sepharose beads). For further discussion regarding  
30   immunoprecipitation protocols see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.16.1.

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (e.g., 8%- 20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (e.g., PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (e.g., PBS-Tween 20), blocking the membrane with primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, blocking the membrane with a secondary antibody (which recognizes the primary antibody, e.g., an anti-human antibody) conjugated to an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) or radioactive molecule (e.g.,  $^{32}\text{P}$  or  $^{125}\text{I}$ ) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding western blot protocols see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1.

ELISAs comprise preparing antigen, coating the well of a 96 well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of the antigen of interest to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1.

The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g.,  $^3\text{H}$  or  $^{125}\text{I}$ ) with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody of interest for a particular antigen and the binding off-rates can be determined from the data by scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, the antigen is incubated with antibody of interest conjugated to a labeled compound (e.g.,  $^3\text{H}$  or  $^{125}\text{I}$ ) in the presence of increasing amounts of an unlabeled second antibody.

#### Therapeutic Uses Of Antibodies

The present invention is further directed to antibody-based therapies which involve administering antibodies of the invention to an animal, preferably a mammal, and most preferably a human, patient for treating one or more of the disclosed diseases, disorders, or conditions. Therapeutic compounds of the invention include, but are not limited to, antibodies of the invention (including fragments, analogs and derivatives thereof as described herein) and nucleic acids encoding antibodies of the invention (including fragments, analogs and derivatives thereof and anti-idiotypic antibodies as described herein). The antibodies of the invention can be used to treat, inhibit or prevent diseases, disorders or conditions associated with aberrant expression and/or activity of a polypeptide of the invention, including, but not limited to, any one or more of the diseases, disorders, or conditions described herein. The treatment and/or prevention of diseases, disorders, or conditions associated with aberrant expression and/or activity of a polypeptide of the invention includes, but is not limited to, alleviating symptoms associated with those diseases, disorders or conditions. Antibodies of the invention may be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

A summary of the ways in which the antibodies of the present invention may be used therapeutically includes binding polynucleotides or polypeptides of the present invention locally or systemically in the body or by direct cytotoxicity of the antibody, e.g. as mediated by complement (CDC) or by effector cells (ADCC). Some

of these approaches are described in more detail below. Armed with the teachings provided herein, one of ordinary skill in the art will know how to use the antibodies of the present invention for diagnostic, monitoring or therapeutic purposes without undue experimentation.

5           The antibodies of this invention may be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hematopoietic tubulin tyrosine ligase proteins (such as, e.g., IL-2, IL-3 and IL-7), for example, which serve to increase the number or activity of effector cells which interact with the antibodies.

10           The antibodies of the invention may be administered alone or in combination with other types of treatments (e.g., radiation therapy, chemotherapy, hormonal therapy, immunotherapy and anti-tumor agents). Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment,  
15           human antibodies, fragments derivatives, analogs, or nucleic acids, are administered to a human patient for therapy or prophylaxis.

          It is preferred to use high affinity and/or potent in vivo inhibiting and/or neutralizing antibodies against polypeptides or polynucleotides of the present invention, fragments or regions thereof, for both immunoassays directed to and  
20           therapy of disorders related to polynucleotides or polypeptides, including fragments thereof, of the present invention. Such antibodies, fragments, or regions, will preferably have an affinity for polynucleotides or polypeptides of the invention, including fragments thereof. Preferred binding affinities include those with a dissociation constant or  $K_d$  less than  $5 \times 10^{-2}$  M,  $10^{-2}$  M,  $5 \times 10^{-3}$  M,  $10^{-3}$  M,  $5 \times$   
25            $10^{-4}$  M,  $10^{-4}$  M,  $5 \times 10^{-5}$  M,  $10^{-5}$  M,  $5 \times 10^{-6}$  M,  $10^{-6}$  M,  $5 \times 10^{-7}$  M,  $10^{-7}$  M,  $5 \times 10^{-8}$  M,  $10^{-8}$  M,  $5 \times 10^{-9}$  M,  $10^{-9}$  M,  $5 \times 10^{-10}$  M,  $10^{-10}$  M,  $5 \times 10^{-11}$  M,  $10^{-11}$  M,  $5 \times 10^{-12}$  M,  $10^{-12}$  M,  $5 \times 10^{-13}$  M,  $10^{-13}$  M,  $5 \times 10^{-14}$  M,  $10^{-14}$  M,  $5 \times 10^{-15}$  M, and  $10^{-15}$  M.

          Antibodies directed against polypeptides of the present invention are useful for  
30           inhibiting allergic reactions in animals. For example, by administering a therapeutically acceptable dose of an antibody, or antibodies, of the present invention,

or a cocktail of the present antibodies, or in combination with other antibodies of varying sources, the animal may not elicit an allergic response to antigens.

Likewise, one could envision cloning the gene encoding an antibody directed against a polypeptide of the present invention, said polypeptide having the potential to elicit an allergic and/or immune response in an organism, and transforming the organism with said antibody gene such that it is expressed (e.g., constitutively, inducibly, etc.) in the organism. Thus, the organism would effectively become resistant to an allergic response resulting from the ingestion or presence of such an immune/allergic reactive polypeptide. Moreover, such a use of the antibodies of the present invention may have particular utility in preventing and/or ameliorating autoimmune diseases and/or disorders, as such conditions are typically a result of antibodies being directed against endogenous proteins. For example, in the instance where the polypeptide of the present invention is responsible for modulating the immune response to auto-antigens, transforming the organism and/or individual with a construct comprising any of the promoters disclosed herein or otherwise known in the art, in addition, to a polynucleotide encoding the antibody directed against the polypeptide of the present invention could effectively inhibit the organism's immune system from eliciting an immune response to the auto-antigen(s). Detailed descriptions of therapeutic and/or gene therapy applications of the present invention are provided elsewhere herein.

Alternatively, antibodies of the present invention could be produced in a plant (e.g., cloning the gene of the antibody directed against a polypeptide of the present invention, and transforming a plant with a suitable vector comprising said gene for constitutive expression of the antibody within the plant), and the plant subsequently ingested by an animal, thereby conferring temporary immunity to the animal for the specific antigen the antibody is directed towards (See, for example, US Patent Nos. 5,914,123 and 6,034,298).

In another embodiment, antibodies of the present invention, preferably polyclonal antibodies, more preferably monoclonal antibodies, and most preferably single-chain antibodies, can be used as a means of inhibiting gene expression of a particular gene, or genes, in a human, mammal, and/or other organism. See, for example, International Publication Number WO 00/05391, published 2/3/00, to Dow

Agrosciences LLC. The application of such methods for the antibodies of the present invention are known in the art, and are more particularly described elsewhere herein.

In yet another embodiment, antibodies of the present invention may be useful for multimerizing the polypeptides of the present invention. For example, certain  
5 proteins may confer enhanced biological activity when present in a multimeric state (i.e., such enhanced activity may be due to the increased effective concentration of such proteins whereby more protein is available in a localized location).

#### Antibody-based Gene Therapy

In a specific embodiment, nucleic acids comprising sequences encoding  
10 antibodies or functional derivatives thereof, are administered to treat, inhibit or prevent a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded  
15 protein that mediates a therapeutic effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., Clinical Pharmacy 12:488-505 (1993); Wu and Wu, Biotherapy 3:87-95 (1991);  
20 Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan, Science 260:926-932 (1993); and Morgan and Anderson, Ann. Rev. Biochem. 62:191-217 (1993); May, TIBTECH 11(5):155-215 (1993). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and  
25 Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

In a preferred aspect, the compound comprises nucleic acid sequences encoding an antibody, said nucleic acid sequences being part of expression vectors that express the antibody or fragments or chimeric proteins or heavy or light chains  
30 thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue- specific. In another particular embodiment,



nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody encoding nucleic acids (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989). In specific  
5 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989). In specific embodiments, the expressed antibody molecule is a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

Delivery of the nucleic acids into a patient may be either direct, in which case  
10 the patient is directly exposed to the nucleic acid or nucleic acid- carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids in vitro, then transplanted into the patient. These two approaches are known, respectively, as in vivo or ex vivo gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered  
15 in vivo, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, e.g., by infection using defective or attenuated retrovirals or other viral vectors (see U.S. Patent No. 4,980,286), or by direct injection  
20 of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, e.g., Wu and Wu,  
25 J. Biol. Chem.. 262:4429-4432 (1987)) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted in vivo for cell specific uptake  
30 and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180; WO 92/22635; WO92/20316; WO93/14188, WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell

DNA for expression, by homologous recombination (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989)).

In a specific embodiment, viral vectors that contains nucleic acid sequences encoding an antibody of the invention are used. For example, a retroviral vector can be used (see Miller et al., Meth. Enzymol. 217:581-599 (1993)). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding the antibody to be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., Biotherapy 6:291-302 (1994), which describes the use of a retroviral vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., J. Clin. Invest. 93:644-651 (1994); Kiem et al., Blood 83:1467-1473 (1994); Salmons and Gunzberg, Human Gene Therapy 4:129-141 (1993); and Grossman and Wilson, Curr. Opin. in Genetics and Devel. 3:110-114 (1993).

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, Current Opinion in Genetics and Development 3:499-503 (1993) present a review of adenovirus-based gene therapy. Bout et al., Human Gene Therapy 5:3-10 (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., Science 252:431-434 (1991); Rosenfeld et al., Cell 68:143- 155 (1992); Mastrangeli et al., J. Clin. Invest. 91:225-234 (1993); PCT Publication WO94/12649; and Wang, et al., Gene Therapy 2:775-783 (1995). In a preferred embodiment, adenovirus vectors are used.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., Proc. Soc. Exp. Biol. Med. 204:289-300 (1993); U.S. Patent No. 5,436,146).

5 Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

10 In this embodiment, the nucleic acid is introduced into a cell prior to administration in vivo of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene  
15 transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, e.g., Loeffler and Behr, Meth. Enzymol. 217:599-618 (1993); Cohen et al., Meth. Enzymol. 217:618-644 (1993); Cline, Pharmac. Ther. 29:69-92m (1985) and may be used in accordance with the present invention, provided that the necessary  
20 developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various  
25 methods known in the art. Recombinant blood cells (e.g., hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy  
30 encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as Tlymphocytes, Blymphocytes, monocytes, macrophages,

neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

5 In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

10 In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered in vivo for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained in vitro can potentially be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598; Stemple and Anderson, Cell 71:973-985 (1992); Rheinwald, Meth. Cell Bio. 21A:229 (1980); and Pittelkow and Scott, Mayo Clinic Proc. 61:771 (1986)).

15 In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription. Demonstration of Therapeutic or Prophylactic Activity

20 The compounds or pharmaceutical compositions of the invention are preferably tested in vitro, and then in vivo for the desired therapeutic or prophylactic activity, prior to use in humans. For example, in vitro assays to demonstrate the therapeutic or prophylactic utility of a compound or pharmaceutical composition include, the effect of a compound on a cell line or a patient tissue sample. The effect  
25 of the compound or composition on the cell line and/or tissue sample can be determined utilizing techniques known to those of skill in the art including, but not limited to, rosette formation assays and cell lysis assays. In accordance with the invention, in vitro assays which can be used to determine whether administration of a specific compound is indicated, include in vitro cell culture assays in which a patient  
30 tissue sample is grown in culture, and exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed.

## Therapeutic/Prophylactic Administration and Compositions

The invention provides methods of treatment, inhibition and prophylaxis by administration to a subject of an effective amount of a compound or pharmaceutical composition of the invention, preferably an antibody of the invention. In a preferred aspect, the compound is substantially purified (e.g., substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid or an immunoglobulin are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

Various delivery systems are known and can be used to administer a compound of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e.g., Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987)), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compounds or compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion

during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when  
5 administering a protein, including an antibody, of the invention, care must be taken to use materials to which the protein does not absorb.

In another embodiment, the compound or composition can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365 (1989); Lopez-Berestein, *ibid.*, pp.  
10 317-327; see generally *ibid.*)

In yet another embodiment, the compound or composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery*  
15 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev.*  
20 *Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp.  
25 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote  
30 expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by

use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., Proc. Natl. Acad. Sci. USA 88:1864-1868 (1991)), etc. Alternatively, a  
5 nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term  
10 "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and  
15 oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients  
20 include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion,  
25 tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical  
30 carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide

the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment, inhibition and prevention of a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention can be determined by standard clinical techniques. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

For antibodies, the dosage administered to a patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a



patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention may be reduced by enhancing uptake and tissue penetration (e.g., into the brain) of the antibodies by modifications such as, for example, lipidation.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

#### Diagnosis and Imaging With Antibodies

Labeled antibodies, and derivatives and analogs thereof, which specifically bind to a polypeptide of interest can be used for diagnostic purposes to detect, diagnose, or monitor diseases, disorders, and/or conditions associated with the aberrant expression and/or activity of a polypeptide of the invention. The invention provides for the detection of aberrant expression of a polypeptide of interest, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of aberrant expression.

The invention provides a diagnostic assay for diagnosing a disorder, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of a particular disorder. With respect to cancer, the presence of a relatively high amount of transcript in biopsied

tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

Antibodies of the invention can be used to assay protein levels in a biological sample using classical immunohistological methods known to those of skill in the art (e.g., see Jalkanen, et al., J. Cell. Biol. 101:976-985 (1985); Jalkanen, et al., J. Cell Biol. 105:3087-3096 (1987)). Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase; radioisotopes, such as iodine ( $^{125}\text{I}$ ,  $^{121}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{112}\text{In}$ ), and technetium ( $^{99}\text{Tc}$ ); luminescent labels, such as luminol; and fluorescent labels, such as fluorescein and rhodamine, and biotin.

One aspect of the invention is the detection and diagnosis of a disease or disorder associated with aberrant expression of a polypeptide of interest in an animal, preferably a mammal and most preferably a human. In one embodiment, diagnosis comprises: a) administering (for example, parenterally, subcutaneously, or intraperitoneally) to a subject an effective amount of a labeled molecule which specifically binds to the polypeptide of interest; b) waiting for a time interval following the administering for permitting the labeled molecule to preferentially concentrate at sites in the subject where the polypeptide is expressed (and for unbound labeled molecule to be cleared to background level); c) determining background level; and d) detecting the labeled molecule in the subject, such that detection of labeled molecule above the background level indicates that the subject has a particular disease or disorder associated with aberrant expression of the polypeptide of interest. Background level can be determined by various methods including, comparing the amount of labeled molecule detected to a standard value previously determined for a particular system.

It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce

diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of <sup>99m</sup>Tc. The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain the specific protein. In vivo tumor  
5 imaging is described in S.W. Burchiel et al., "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments." (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982).

Depending on several variables, including the type of label used and the mode  
10 of administration, the time interval following the administration for permitting the labeled molecule to preferentially concentrate at sites in the subject and for unbound labeled molecule to be cleared to background level is 6 to 48 hours or 6 to 24 hours or 6 to 12 hours. In another embodiment the time interval following administration is 5 to 20 days or 5 to 10 days.

15 In an embodiment, monitoring of the disease or disorder is carried out by repeating the method for diagnosing the disease or disease, for example, one month after initial diagnosis, six months after initial diagnosis, one year after initial diagnosis, etc.

Presence of the labeled molecule can be detected in the patient using methods  
20 known in the art for in vivo scanning. These methods depend upon the type of label used. Skilled artisans will be able to determine the appropriate method for detecting a particular label. Methods and devices that may be used in the diagnostic methods of the invention include, but are not limited to, computed tomography (CT), whole body scan such as position emission tomography (PET), magnetic resonance imaging  
25 (MRI), and sonography.

In a specific embodiment, the molecule is labeled with a radioisotope and is detected in the patient using a radiation responsive surgical instrument (Thurston et al., U.S. Patent No. 5,441,050). In another embodiment, the molecule is labeled with a fluorescent compound and is detected in the patient using a fluorescence responsive  
30 scanning instrument. In another embodiment, the molecule is labeled with a positron emitting metal and is detected in the patent using positron emission-tomography. In

yet another embodiment, the molecule is labeled with a paramagnetic label and is detected in a patient using magnetic resonance imaging (MRI).

#### Kits

The present invention provides kits that can be used in the above methods. In one embodiment, a kit comprises an antibody of the invention, preferably a purified antibody, in one or more containers. In a specific embodiment, the kits of the present invention contain a substantially isolated polypeptide comprising an epitope which is specifically immunoreactive with an antibody included in the kit. Preferably, the kits of the present invention further comprise a control antibody which does not react with the polypeptide of interest. In another specific embodiment, the kits of the present invention contain a means for detecting the binding of an antibody to a polypeptide of interest (e.g., the antibody may be conjugated to a detectable substrate such as a fluorescent compound, an enzymatic substrate, a radioactive compound or a luminescent compound, or a second antibody which recognizes the first antibody may be conjugated to a detectable substrate).

In another specific embodiment of the present invention, the kit is a diagnostic kit for use in screening serum containing antibodies specific against proliferative and/or cancerous polynucleotides and polypeptides. Such a kit may include a control antibody that does not react with the polypeptide of interest. Such a kit may include a substantially isolated polypeptide antigen comprising an epitope which is specifically immunoreactive with at least one anti-polypeptide antigen antibody. Further, such a kit includes means for detecting the binding of said antibody to the antigen (e.g., the antibody may be conjugated to a fluorescent compound such as fluorescein or rhodamine which can be detected by flow cytometry). In specific embodiments, the kit may include a recombinantly produced or chemically synthesized polypeptide antigen. The polypeptide antigen of the kit may also be attached to a solid support.

In a more specific embodiment the detecting means of the above-described kit includes a solid support to which said polypeptide antigen is attached. Such a kit may also include a non-attached reporter-labeled anti-human antibody. In this embodiment, binding of the antibody to the polypeptide antigen can be detected by binding of the said reporter-labeled antibody.

In an additional embodiment, the invention includes a diagnostic kit for use in screening serum containing antigens of the polypeptide of the invention. The diagnostic kit includes a substantially isolated antibody specifically immunoreactive with polypeptide or polynucleotide antigens, and means for detecting the binding of the polynucleotide or polypeptide antigen to the antibody. In one embodiment, the antibody is attached to a solid support. In a specific embodiment, the antibody may be a monoclonal antibody. The detecting means of the kit may include a second, labeled monoclonal antibody. Alternatively, or in addition, the detecting means may include a labeled, competing antigen.

10 In one diagnostic configuration, test serum is reacted with a solid phase reagent having a surface-bound antigen obtained by the methods of the present invention. After binding with specific antigen antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-antigen antibody on the solid support. The reagent is again washed to remove unbound labeled antibody, and the amount of reporter associated with the reagent is determined. Typically, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric, luminescent or colorimetric substrate (Sigma, St. Louis, MO).

20 The solid surface reagent in the above assay is prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, 96-well plate or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group. Alternatively, streptavidin coated plates can be used in conjunction with biotinylated antigen(s).

Thus, the invention provides an assay system or kit for carrying out this diagnostic method. The kit generally includes a support with surface-bound recombinant antigens, and a reporter-labeled anti-human antibody for detecting surface-bound anti-antigen antibody.

## Fusion Proteins

Any polypeptide of the present invention can be used to generate fusion proteins. For example, the polypeptide of the present invention, when fused to a second protein, can be used as an antigenic tag. Antibodies raised against the polypeptide of the present invention can be used to indirectly detect the second protein by binding to the polypeptide. Moreover, because certain proteins target cellular locations based on trafficking signals, the polypeptides of the present invention can be used as targeting molecules once fused to other proteins.

Examples of domains that can be fused to polypeptides of the present invention include not only heterologous signal sequences, but also other heterologous functional regions. The fusion does not necessarily need to be direct, but may occur through linker sequences.

Moreover, fusion proteins may also be engineered to improve characteristics of the polypeptide of the present invention. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence during purification from the host cell or subsequent handling and storage. Peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. Similarly, peptide cleavage sites can be introduced in-between such peptide moieties, which could additionally be subjected to protease activity to remove said peptide(s) from the protein of the present invention. The addition of peptide moieties, including peptide cleavage sites, to facilitate handling of polypeptides are familiar and routine techniques in the art.

Moreover, polypeptides of the present invention, including fragments, and specifically epitopes, can be combined with parts of the constant domain of immunoglobulins (IgA, IgE, IgG, IgM) or portions thereof (CH1, CH2, CH3, and any combination thereof, including both entire domains and portions thereof), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life in vivo. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP A 394,827; Traunecker et al., Nature 331:84-86 (1988).)

Fusion proteins having disulfide-linked dimeric structures (due to the IgG) can also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis et al., J. Biochem. 270:3958-3964 (1995).)

5           Similarly, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of the constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP-A 0232 262.) Alternatively,  
10 deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. (See, D.  
15 Bennett et al., J. Molecular Recognition 8:52-58 (1995); K. Johanson et al., J. Biol. Chem. 270:9459-9471 (1995).)

Moreover, the polypeptides of the present invention can be fused to marker sequences (also referred to as "tags"). Due to the availability of antibodies specific to such "tags", purification of the fused polypeptide of the invention, and/or its  
20 identification is significantly facilitated since antibodies specific to the polypeptides of the invention are not required. Such purification may be in the form of an affinity purification whereby an anti-tag antibody or another type of affinity matrix (e.g., anti-tag antibody attached to the matrix of a flow-thru column) that binds to the epitope tag is present. In preferred embodiments, the marker amino acid sequence is a hexa-  
25 histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz et al., Proc. Natl. Acad. Sci. USA 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Another peptide tag useful for purification, the "HA" tag, corresponds to an  
30 epitope derived from the influenza hemagglutinin protein. (Wilson et al., Cell 37:767 (1984)).

The skilled artisan would acknowledge the existence of other “tags” which could be readily substituted for the tags referred to supra for purification and/or identification of polypeptides of the present invention (Jones C., et al., J Chromatogr A. 707(1):3-22 (1995)). For example, the c-myc tag and the 8F9, 3C7, 6E10, G4m B7  
5 and 9E10 antibodies thereto (Evan et al., Molecular and Cellular Biology 5:3610-3616 (1985)); the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky et al., Protein Engineering, 3(6):547-553 (1990), the Flag-peptide – i.e., the octapeptide sequence DYKDDDDK (SEQ ID NO:61), (Hopp et al., Biotech. 6:1204-1210 (1988); the KT3 epitope peptide (Martin et al., Science, 255:192-194 (1992)); a-  
10 tubulin epitope peptide (Skinner et al., J. Biol. Chem., 266:15136-15166, (1991)); the T7 gene 10 protein peptide tag (Lutz-Freyermuth et al., Proc. Natl. Sci. USA, 87:6363-6397 (1990)), the FITC epitope (Zymed, Inc.), the GFP epitope (Zymed, Inc.), and the Rhodamine epitope (Zymed, Inc.).

The present invention also encompasses the attachment of up to nine codons  
15 encoding a repeating series of up to nine arginine amino acids to the coding region of a polynucleotide of the present invention. The invention also encompasses chemically derivitizing a polypeptide of the present invention with a repeating series of up to nine arginine amino acids. Such a tag, when attached to a polypeptide, has recently been shown to serve as a universal pass, allowing compounds access to the interior of cells  
20 without additional derivitization or manipulation (Wender, P., et al., unpublished data).

Protein fusions involving polypeptides of the present invention, including fragments and/or variants thereof, can be used for the following, non-limiting examples, subcellular localization of proteins, determination of protein-protein  
25 interactions via immunoprecipitation, purification of proteins via affinity chromatography, functional and/or structural characterization of protein. The present invention also encompasses the application of hapten specific antibodies for any of the uses referenced above for epitope fusion proteins. For example, the polypeptides of the present invention could be chemically derivatized to attach hapten molecules  
30 (e.g., DNP, (Zymed, Inc.)). Due to the availability of monoclonal antibodies specific to such haptens, the protein could be readily purified using immunoprecipitation, for example.



Polypeptides of the present invention, including fragments and/or variants thereof, in addition to, antibodies directed against such polypeptides, fragments, and/or variants, may be fused to any of a number of known, and yet to be determined, toxins, such as ricin, saporin (Mashiba H, et al., Ann. N. Y. Acad. Sci. 1999;886:233-5), or HC toxin (Tonukari NJ, et al., Plant Cell. 2000 Feb;12(2):237-248), for example. Such fusions could be used to deliver the toxins to desired tissues for which a ligand or a protein capable of binding to the polypeptides of the invention exists.

The invention encompasses the fusion of antibodies directed against polypeptides of the present invention, including variants and fragments thereof, to said toxins for delivering the toxin to specific locations in a cell, to specific tissues, and/or to specific species. Such bifunctional antibodies are known in the art, though a review describing additional advantageous fusions, including citations for methods of production, can be found in P.J. Hudson, Curr. Opp. In. Imm. 11:548-557, (1999); this publication, in addition to the references cited therein, are hereby incorporated by reference in their entirety herein. In this context, the term "toxin" may be expanded to include any heterologous protein, a small molecule, radionucleotides, cytotoxic drugs, liposomes, adhesion molecules, glycoproteins, ligands, cell or tissue-specific ligands, enzymes, of bioactive agents, biological response modifiers, anti-fungal agents, hormones, steroids, vitamins, peptides, peptide analogs, anti-allergenic agents, anti-tubercular agents, anti-viral agents, antibiotics, anti-protozoan agents, chelates, radioactive particles, radioactive ions, X-ray contrast agents, monoclonal antibodies, polyclonal antibodies and genetic material. In view of the present disclosure, one skilled in the art could determine whether any particular "toxin" could be used in the compounds of the present invention. Examples of suitable "toxins" listed above are exemplary only and are not intended to limit the "toxins" that may be used in the present invention.

Thus, any of these above fusions can be engineered using the polynucleotides or the polypeptides of the present invention.

#### Vectors, Host Cells, and Protein Production

The present invention also relates to vectors containing the polynucleotide of the present invention, host cells, and the production of polypeptides by recombinant techniques. The vector may be, for example, a phage, plasmid, viral, or retroviral

vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

5 The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

10 The polynucleotide insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli* lac, trp, phoA and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding  
15 portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin  
20 resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells (e.g., *Saccharomyces cerevisiae* or *Pichia pastoris* (ATCC Accession No. 201178));  
25 insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, 293, and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from QIAGEN, Inc.; pBluescript vectors, Phagescript vectors, pNH8A,  
30 pNH16a, pNH18A, pNH46A, available from Stratagene Cloning Systems, Inc.; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia Biotech, Inc. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1

and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Preferred expression vectors for use in yeast systems include, but are not limited to pYES2, pYD1, pTEF1/Zeo, pYES2/GS, pPICZ, pGAPZ, pGAPZalph, pPIC9, pPIC3.5, pHIL-D2, pHIL-S1, pPIC3.5K, pPIC9K, and PAO815 (all available  
5 from Invitrogen, Carlsbad, CA). Other suitable vectors will be readily apparent to the skilled artisan.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. Such methods  
10 are described in many standard laboratory manuals, such as Davis et al., Basic Methods In Molecular Biology (1986). It is specifically contemplated that the polypeptides of the present invention may in fact be expressed by a host cell lacking a recombinant vector.

A polypeptide of this invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for  
15 purification.  
20

Polypeptides of the present invention, and preferably the secreted form, can also be recovered from: products purified from natural sources, including bodily fluids, tissues and cells, whether directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a  
25 prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect, and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-  
30 mediated processes. Thus, it is well known in the art that the N-terminal methionine encoded by the translation initiation codon generally is removed with high efficiency from any protein after translation in all eukaryotic cells. While the N-terminal

methionine on most proteins also is efficiently removed in most prokaryotes, for some proteins, this prokaryotic removal process is inefficient, depending on the nature of the amino acid to which the N-terminal methionine is covalently linked.

In one embodiment, the yeast *Pichia pastoris* is used to express the polypeptide of the present invention in a eukaryotic system. *Pichia pastoris* is a methylotrophic yeast which can metabolize methanol as its sole carbon source. A main step in the methanol metabolization pathway is the oxidation of methanol to formaldehyde using O<sub>2</sub>. This reaction is catalyzed by the enzyme alcohol oxidase. In order to metabolize methanol as its sole carbon source, *Pichia pastoris* must generate high levels of alcohol oxidase due, in part, to the relatively low affinity of alcohol oxidase for O<sub>2</sub>. Consequently, in a growth medium depending on methanol as a main carbon source, the promoter region of one of the two alcohol oxidase genes (AOX1) is highly active. In the presence of methanol, alcohol oxidase produced from the AOX1 gene comprises up to approximately 30% of the total soluble protein in *Pichia pastoris*. See, Ellis, S.B., et al., *Mol. Cell. Biol.* 5:1111-21 (1985); Koutz, P.J, et al., *Yeast* 5:167-77 (1989); Tschopp, J.F., et al., *Nucl. Acids Res.* 15:3859-76 (1987). Thus, a heterologous coding sequence, such as, for example, a polynucleotide of the present invention, under the transcriptional regulation of all or part of the AOX1 regulatory sequence is expressed at exceptionally high levels in *Pichia* yeast grown in the presence of methanol.

In one example, the plasmid vector pPIC9K is used to express DNA encoding a polypeptide of the invention, as set forth herein, in a *Pichea* yeast system essentially as described in "Pichia Protocols: Methods in Molecular Biology" D.R. Higgins and J. Cregg, eds. The Humana Press, Totowa, NJ, 1998. This expression vector allows expression and secretion of a protein of the invention by virtue of the strong AOX1 promoter linked to the *Pichia pastoris* alkaline phosphatase (PHO) secretory signal peptide (i.e., leader) located upstream of a multiple cloning site.

Many other yeast vectors could be used in place of pPIC9K, such as, pYES2, pYD1, pTEF1/Zeo, pYES2/GS, pPICZ, pGAPZ, pGAPZalpha, pPIC9, pPIC3.5, pHIL-D2, pHIL-S1, pPIC3.5K, and PAO815, as one skilled in the art would readily appreciate, as long as the proposed expression construct provides appropriately

located signals for transcription, translation, secretion (if desired), and the like, including an in-frame AUG, as required.

In another embodiment, high-level expression of a heterologous coding sequence, such as, for example, a polynucleotide of the present invention, may be achieved by cloning the heterologous polynucleotide of the invention into an expression vector such as, for example, pGAPZ or pGAPZalpha, and growing the yeast culture in the absence of methanol.

In addition to encompassing host cells containing the vector constructs discussed herein, the invention also encompasses primary, secondary, and immortalized host cells of vertebrate origin, particularly mammalian origin, that have been engineered to delete or replace endogenous genetic material (e.g., coding sequence), and/or to include genetic material (e.g., heterologous polynucleotide sequences) that is operably associated with the polynucleotides of the invention, and which activates, alters, and/or amplifies endogenous polynucleotides. For example, techniques known in the art may be used to operably associate heterologous control regions (e.g., promoter and/or enhancer) and endogenous polynucleotide sequences via homologous recombination, resulting in the formation of a new transcription unit (see, e.g., U.S. Patent No. 5,641,670, issued June 24, 1997; U.S. Patent No. 5,733,761, issued March 31, 1998; International Publication No. WO 96/29411, published September 26, 1996; International Publication No. WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); and Zijlstra et al., Nature 342:435-438 (1989), the disclosures of each of which are incorporated by reference in their entireties).

In addition, polypeptides of the invention can be chemically synthesized using techniques known in the art (e.g., see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y., and Hunkapiller et al., Nature, 310:105-111 (1984)). For example, a polypeptide corresponding to a fragment of a polypeptide sequence of the invention can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the polypeptide sequence. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid,  $\alpha$ -amino isobutyric acid, 4-

aminobutyric acid, Abu, 2-amino butyric acid, g-Abu, e-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, b-alanine, fluoro-  
5 amino acids, designer amino acids such as b-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

The invention encompasses polypeptides which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation,  
10 amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH<sub>4</sub>; acetylation, formylation, oxidation, reduction;  
15 metabolic synthesis in the presence of tunicamycin; etc.

Additional post-translational modifications encompassed by the invention include, for example, e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and  
20 addition or deletion of an N-terminal methionine residue as a result of prokaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein, the addition of epitope tagged peptide fragments (e.g., FLAG, HA, GST, thioredoxin, maltose binding protein, etc.), attachment of affinity tags such  
25 as biotin and/or streptavidin, the covalent attachment of chemical moieties to the amino acid backbone, N- or C-terminal processing of the polypeptides ends (e.g., proteolytic processing), deletion of the N-terminal methionine residue, etc.

Also provided by the invention are chemically modified derivatives of the polypeptides of the invention which may provide additional advantages such as  
30 increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U.S. Patent NO: 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene

glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

5           The invention further encompasses chemical derivitization of the polypeptides of the present invention, preferably where the chemical is a hydrophilic polymer residue. Exemplary hydrophilic polymers, including derivatives, may be those that include polymers in which the repeating units contain one or more hydroxy groups (polyhydroxy polymers), including, for example, poly(vinyl alcohol); polymers in  
10       which the repeating units contain one or more amino groups (polyamine polymers), including, for example, peptides, polypeptides, proteins and lipoproteins, such as albumin and natural lipoproteins; polymers in which the repeating units contain one or more carboxy groups (polycarboxy polymers), including, for example, carboxymethylcellulose, alginic acid and salts thereof, such as sodium and calcium  
15       alginate, glycosaminoglycans and salts thereof, including salts of hyaluronic acid, phosphorylated and sulfonated derivatives of carbohydrates, genetic material, such as interleukin-2 and interferon, and phosphorothioate oligomers; and polymers in which the repeating units contain one or more saccharide moieties (polysaccharide polymers), including, for example, carbohydrates.

20           The molecular weight of the hydrophilic polymers may vary, and is generally about 50 to about 5,000,000, with polymers having a molecular weight of about 100 to about 50,000 being preferred. The polymers may be branched or unbranched. More preferred polymers have a molecular weight of about 150 to about 10,000, with molecular weights of 200 to about 8,000 being even more preferred.

25           For polyethylene glycol, the preferred molecular weight is between about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release  
30       desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

Additional preferred polymers which may be used to derivatize polypeptides of the invention, include, for example, poly(ethylene glycol) (PEG), poly(vinylpyrrolidone), polyoxomers, polysorbate and poly(vinyl alcohol), with PEG polymers being particularly preferred. Preferred among the PEG polymers are PEG polymers having a molecular weight of from about 100 to about 10,000. More preferably, the PEG polymers have a molecular weight of from about 200 to about 8,000, with PEG 2,000, PEG 5,000 and PEG 8,000, which have molecular weights of 2,000, 5,000 and 8,000, respectively, being even more preferred. Other suitable hydrophilic polymers, in addition to those exemplified above, will be readily apparent to one skilled in the art based on the present disclosure. Generally, the polymers used may include polymers that can be attached to the polypeptides of the invention via alkylation or acylation reactions.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, e.g., EP 0 401 384, herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol. 20:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

One may specifically desire proteins chemically modified at the N-terminus. Using polyethylene glycol as an illustration of the present composition, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (polypeptide) molecules in the reaction mix, the type of pegylation reaction to be



performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules.

5 Selective proteins chemically modified at the N-terminus modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminus) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl  
10 group containing polymer is achieved.

As with the various polymers exemplified above, it is contemplated that the polymeric residues may contain functional groups in addition, for example, to those typically involved in linking the polymeric residues to the polypeptides of the present invention. Such functionalities include, for example, carboxyl, amine, hydroxy and  
15 thiol groups. These functional groups on the polymeric residues can be further reacted, if desired, with materials that are generally reactive with such functional groups and which can assist in targeting specific tissues in the body including, for example, diseased tissue. Exemplary materials which can be reacted with the additional functional groups include, for example, proteins, including antibodies,  
20 carbohydrates, peptides, glycopeptides, glycolipids, lectins, and nucleosides.

In addition to residues of hydrophilic polymers, the chemical used to derivatize the polypeptides of the present invention can be a saccharide residue. Exemplary saccharides which can be derived include, for example, monosaccharides or sugar alcohols, such as erythrose, threose, ribose, arabinose, xylose, lyxose,  
25 fructose, sorbitol, mannitol and sedoheptulose, with preferred monosaccharides being fructose, mannose, xylose, arabinose, mannitol and sorbitol; and disaccharides, such as lactose, sucrose, maltose and cellobiose. Other saccharides include, for example, inositol and ganglioside head groups. Other suitable saccharides, in addition to those exemplified above, will be readily apparent to one skilled in the art based on the  
30 present disclosure. Generally, saccharides which may be used for derivitization include saccharides that can be attached to the polypeptides of the invention via alkylation or acylation reactions.

Moreover, the invention also encompasses derivitization of the polypeptides of the present invention, for example, with lipids (including cationic, anionic, polymerized, charged, synthetic, saturated, unsaturated, and any combination of the above, etc.). stabilizing agents.

5       The invention encompasses derivitization of the polypeptides of the present invention, for example, with compounds that may serve a stabilizing function (e.g., to increase the polypeptides half-life in solution, to make the polypeptides more water soluble, to increase the polypeptides hydrophilic or hydrophobic character, etc.). Polymers useful as stabilizing materials may be of natural, semi-synthetic (modified  
10 natural) or synthetic origin. Exemplary natural polymers include naturally occurring polysaccharides, such as, for example, arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans (such as, for example, inulin), levan, fucoidan, carrageenan, galatocarlose, pectic acid, pectins, including amylose, pullulan, glycogen, amylopectin, cellulose, dextran, dextrin, dextrose, glucose,  
15 polyglucose, polydextrose, pustulan, chitin, agarose, keratin, chondroitin, dermatan, hyaluronic acid, alginic acid, xanthin gum, starch and various other natural homopolymer or heteropolymers, such as those containing one or more of the following aldoses, ketoses, acids or amines: erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, dextrose, mannose, gulose, idose, galactose,  
20 talose, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, mannitol, sorbitol, lactose, sucrose, trehalose, maltose, cellobiose, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, glucosamine, galactosamine, and neuraminic acid, and naturally  
25 occurring derivatives thereof Accordingly, suitable polymers include, for example, proteins, such as albumin, polyalginates, and polylactide-coglycolide polymers. Exemplary semi-synthetic polymers include carboxymethylcellulose, hydroxymethylcellulose, hydroxypropylmethylcellulose, methylcellulose, and methoxycellulose. Exemplary synthetic polymers include polyphosphazenes,  
30 hydroxyapatites, fluoroapatite polymers, polyethylenes (such as, for example, polyethylene glycol (including for example, the class of compounds referred to as Pluronics.RTM., commercially available from BASF, Parsippany, N.J.),

polyoxyethylene, and polyethylene terephthalate), polypropylenes (such as, for example, polypropylene glycol), polyurethanes (such as, for example, polyvinyl alcohol (PVA), polyvinyl chloride and polyvinylpyrrolidone), polyamides including nylon, polystyrene, polylactic acids, fluorinated hydrocarbon polymers, fluorinated carbon polymers (such as, for example, polytetrafluoroethylene), acrylate, methacrylate, and polymethylmethacrylate, and derivatives thereof. Methods for the preparation of derivatized polypeptides of the invention which employ polymers as stabilizing compounds will be readily apparent to one skilled in the art, in view of the present disclosure, when coupled with information known in the art, such as that described and referred to in Unger, U.S. Pat. No. 5,205,290, the disclosure of which is hereby incorporated by reference herein in its entirety.

Moreover, the invention encompasses additional modifications of the polypeptides of the present invention. Such additional modifications are known in the art, and are specifically provided, in addition to methods of derivitization, etc., in US Patent No. 6,028,066, which is hereby incorporated in its entirety herein.

The polypeptides of the invention may be in monomers or multimers (i.e., dimers, trimers, tetramers and higher multimers). Accordingly, the present invention relates to monomers and multimers of the polypeptides of the invention, their preparation, and compositions (preferably, Therapeutics) containing them. In specific embodiments, the polypeptides of the invention are monomers, dimers, trimers or tetramers. In additional embodiments, the multimers of the invention are at least dimers, at least trimers, or at least tetramers.

Multimers encompassed by the invention may be homomers or heteromers. As used herein, the term homomer, refers to a multimer containing only polypeptides corresponding to the amino acid sequence of SEQ ID NO:2 or encoded by the cDNA contained in a deposited clone (including fragments, variants, splice variants, and fusion proteins, corresponding to these polypeptides as described herein). These homomers may contain polypeptides having identical or different amino acid sequences. In a specific embodiment, a homomer of the invention is a multimer containing only polypeptides having an identical amino acid sequence. In another specific embodiment, a homomer of the invention is a multimer containing polypeptides having different amino acid sequences. In specific embodiments, the

multimer of the invention is a homodimer (e.g., containing polypeptides having identical or different amino acid sequences) or a homotrimer (e.g., containing polypeptides having identical and/or different amino acid sequences). In additional embodiments, the homomeric multimer of the invention is at least a homodimer, at  
5 least a homotrimer, or at least a homotetramer.

As used herein, the term heteromer refers to a multimer containing one or more heterologous polypeptides (i.e., polypeptides of different proteins) in addition to the polypeptides of the invention. In a specific embodiment, the multimer of the invention is a heterodimer, a heterotrimer, or a heterotetramer. In additional  
10 embodiments, the heteromeric multimer of the invention is at least a heterodimer, at least a heterotrimer, or at least a heterotetramer.

Multimers of the invention may be the result of hydrophobic, hydrophilic, ionic and/or covalent associations and/or may be indirectly linked, by for example, liposome formation. Thus, in one embodiment, multimers of the invention, such as,  
15 for example, homodimers or homotrimers, are formed when polypeptides of the invention contact one another in solution. In another embodiment, heteromultimers of the invention, such as, for example, heterotrimers or heterotetramers, are formed when polypeptides of the invention contact antibodies to the polypeptides of the invention (including antibodies to the heterologous polypeptide sequence in a fusion  
20 protein of the invention) in solution. In other embodiments, multimers of the invention are formed by covalent associations with and/or between the polypeptides of the invention. Such covalent associations may involve one or more amino acid residues contained in the polypeptide sequence (e.g., that recited in the sequence listing, or contained in the polypeptide encoded by a deposited clone). In one instance,  
25 the covalent associations are cross-linking between cysteine residues located within the polypeptide sequences which interact in the native (i.e., naturally occurring) polypeptide. In another instance, the covalent associations are the consequence of chemical or recombinant manipulation. Alternatively, such covalent associations may involve one or more amino acid residues contained in the heterologous polypeptide  
30 sequence in a fusion protein of the invention.

In one example, covalent associations are between the heterologous sequence contained in a fusion protein of the invention (see, e.g., US Patent Number

5,478,925). In a specific example, the covalent associations are between the heterologous sequence contained in an Fc fusion protein of the invention (as described herein). In another specific example, covalent associations of fusion proteins of the invention are between heterologous polypeptide sequence from another protein that is  
5 capable of forming covalently associated multimers, such as for example, osteoprotegerin (see, e.g., International Publication NO: WO 98/49305, the contents of which are herein incorporated by reference in its entirety). In another embodiment, two or more polypeptides of the invention are joined through peptide linkers. Examples include those peptide linkers described in U.S. Pat. No. 5,073,627 (hereby  
10 incorporated by reference). Proteins comprising multiple polypeptides of the invention separated by peptide linkers may be produced using conventional recombinant DNA technology.

Another method for preparing multimer polypeptides of the invention involves use of polypeptides of the invention fused to a leucine zipper or isoleucine zipper  
15 polypeptide sequence. Leucine zipper and isoleucine zipper domains are polypeptides that promote multimerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., Science 240:1759, (1988)), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives  
20 thereof that dimerize or trimerize. Examples of leucine zipper domains suitable for producing soluble multimeric proteins of the invention are those described in PCT application WO 94/10308, hereby incorporated by reference. Recombinant fusion proteins comprising a polypeptide of the invention fused to a polypeptide sequence that dimerizes or trimerizes in solution are expressed in suitable host cells, and the  
25 resulting soluble multimeric fusion protein is recovered from the culture supernatant using techniques known in the art.

Trimeric polypeptides of the invention may offer the advantage of enhanced biological activity. Preferred leucine zipper moieties and isoleucine moieties are those that preferentially form trimers. One example is a leucine zipper derived from lung  
30 surfactant protein D (SPD), as described in Hoppe et al. (FEBS Letters 344:191, (1994)) and in U.S. patent application Ser. No. 08/446,922, hereby incorporated by

reference. Other peptides derived from naturally occurring trimeric proteins may be employed in preparing trimeric polypeptides of the invention.

In another example, proteins of the invention are associated by interactions between Flag® polypeptide sequence contained in fusion proteins of the invention containing Flag® polypeptide sequence. In a further embodiment, associations  
5 proteins of the invention are associated by interactions between heterologous polypeptide sequence contained in Flag® fusion proteins of the invention and anti-Flag® antibody.

The multimers of the invention may be generated using chemical techniques  
10 known in the art. For example, polypeptides desired to be contained in the multimers of the invention may be chemically cross-linked using linker molecules and linker molecule length optimization techniques known in the art (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, multimers of the invention may be generated using techniques known in  
15 the art to form one or more inter-molecule cross-links between the cysteine residues located within the sequence of the polypeptides desired to be contained in the multimer (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Further, polypeptides of the invention may be routinely modified by the addition of cysteine or biotin to the C terminus or N-terminus of the  
20 polypeptide and techniques known in the art may be applied to generate multimers containing one or more of these modified polypeptides (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, techniques known in the art may be applied to generate liposomes containing the polypeptide components desired to be contained in the multimer of the invention (see,  
25 e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

Alternatively, multimers of the invention may be generated using genetic engineering techniques known in the art. In one embodiment, polypeptides contained in multimers of the invention are produced recombinantly using fusion protein  
30 technology described herein or otherwise known in the art (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In a specific embodiment, polynucleotides coding for a homodimer of the invention are

generated by ligating a polynucleotide sequence encoding a polypeptide of the invention to a sequence encoding a linker polypeptide and then further to a synthetic polynucleotide encoding the translated product of the polypeptide in the reverse orientation from the original C-terminus to the N-terminus (lacking the leader sequence) (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In another embodiment, recombinant techniques described herein or otherwise known in the art are applied to generate recombinant polypeptides of the invention which contain a transmembrane domain (or hydrophobic or signal peptide) and which can be incorporated by membrane reconstitution techniques into liposomes (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

In addition, the polynucleotide insert of the present invention could be operatively linked to "artificial" or chimeric promoters and transcription factors. Specifically, the artificial promoter could comprise, or alternatively consist, of any combination of cis-acting DNA sequence elements that are recognized by trans-acting transcription factors. Preferably, the cis acting DNA sequence elements and trans-acting transcription factors are operable in mammals. Further, the trans-acting transcription factors of such "artificial" promoters could also be "artificial" or chimeric in design themselves and could act as activators or repressors to said "artificial" promoter.

#### Uses of the Polynucleotides

Each of the polynucleotides identified herein can be used in numerous ways as reagents. The following description should be considered exemplary and utilizes known techniques.

The polynucleotides of the present invention are useful for chromosome identification. There exists an ongoing need to identify new chromosome markers, since few chromosome marking reagents, based on actual sequence data (repeat polymorphisms), are presently available. Each polynucleotide of the present invention can be used as a chromosome marker.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the sequences shown in SEQ ID NO:1. Primers can be selected using computer analysis so that primers do not span more than one predicted

exon in the genomic DNA. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the SEQ ID NO:1 will yield an amplified fragment.

5           Similarly, somatic hybrids provide a rapid method of PCR mapping the polynucleotides to particular chromosomes. Three or more clones can be assigned per day using a single thermal cycler. Moreover, sublocalization of the polynucleotides can be achieved with panels of specific chromosome fragments. Other gene mapping strategies that can be used include in situ hybridization, prescreening with labeled  
10 flow-sorted chromosomes, and preselection by hybridization to construct chromosome specific-cDNA libraries.

Precise chromosomal location of the polynucleotides can also be achieved using fluorescence in situ hybridization (FISH) of a metaphase chromosomal spread. This technique uses polynucleotides as short as 500 or 600 bases; however,  
15 polynucleotides 2,000-4,000 bp are preferred. For a review of this technique, see Verma et al., "Human Chromosomes: a Manual of Basic Techniques" Pergamon Press, New York (1988).

For chromosome mapping, the polynucleotides can be used individually (to mark a single chromosome or a single site on that chromosome) or in panels (for  
20 marking multiple sites and/or multiple chromosomes). Preferred polynucleotides correspond to the noncoding regions of the cDNAs because the coding sequences are more likely conserved within gene families, thus increasing the chance of cross hybridization during chromosomal mapping.

Once a polynucleotide has been mapped to a precise chromosomal location,  
25 the physical position of the polynucleotide can be used in linkage analysis. Linkage analysis establishes coinheritance between a chromosomal location and presentation of a particular disease. Disease mapping data are known in the art. Assuming 1 megabase mapping resolution and one gene per 20 kb, a cDNA precisely localized to a chromosomal region associated with the disease could be one of 50-500 potential  
30 causative genes.

Thus, once coinheritance is established, differences in the polynucleotide and the corresponding gene between affected and unaffected organisms can be examined.



First, visible structural alterations in the chromosomes, such as deletions or translocations, are examined in chromosome spreads or by PCR. If no structural alterations exist, the presence of point mutations are ascertained. Mutations observed in some or all affected organisms, but not in normal organisms, indicates that the mutation may cause the disease. However, complete sequencing of the polypeptide and the corresponding gene from several normal organisms is required to distinguish the mutation from a polymorphism. If a new polymorphism is identified, this polymorphic polypeptide can be used for further linkage analysis.

Furthermore, increased or decreased expression of the gene in affected organisms as compared to unaffected organisms can be assessed using polynucleotides of the present invention. Any of these alterations (altered expression, chromosomal rearrangement, or mutation) can be used as a diagnostic or prognostic marker.

Thus, the invention also provides a diagnostic method useful during diagnosis of a disorder, involving measuring the expression level of polynucleotides of the present invention in cells or body fluid from an organism and comparing the measured gene expression level with a standard level of polynucleotide expression level, whereby an increase or decrease in the gene expression level compared to the standard is indicative of a disorder.

By "measuring the expression level of a polynucleotide of the present invention" is intended qualitatively or quantitatively measuring or estimating the level of the polypeptide of the present invention or the level of the mRNA encoding the polypeptide in a first biological sample either directly (e.g., by determining or estimating absolute protein level or mRNA level) or relatively (e.g., by comparing to the polypeptide level or mRNA level in a second biological sample). Preferably, the polypeptide level or mRNA level in the first biological sample is measured or estimated and compared to a standard polypeptide level or mRNA level, the standard being taken from a second biological sample obtained from an individual not having the disorder or being determined by averaging levels from a population of organisms not having a disorder. As will be appreciated in the art, once a standard polypeptide level or mRNA level is known, it can be used repeatedly as a standard for comparison.

By "biological sample" is intended any biological sample obtained from an organism, body fluids, cell line, tissue culture, or other source which contains the polypeptide of the present invention or mRNA. As indicated, biological samples include body fluids (such as the following non-limiting examples, sputum, amniotic fluid, urine, saliva, breast milk, secretions, interstitial fluid, blood, serum, spinal fluid, etc.) which contain the polypeptide of the present invention, and other tissue sources found to express the polypeptide of the present invention. Methods for obtaining tissue biopsies and body fluids from organisms are well known in the art. Where the biological sample is to include mRNA, a tissue biopsy is the preferred source.

The method(s) provided above may Preferably be applied in a diagnostic method and/or kits in which polynucleotides and/or polypeptides are attached to a solid support. In one exemplary method, the support may be a "gene chip" or a "biological chip" as described in US Patents 5,837,832, 5,874,219, and 5,856,174. Further, such a gene chip with polynucleotides of the present invention attached may be used to identify polymorphisms between the polynucleotide sequences, with polynucleotides isolated from a test subject. The knowledge of such polymorphisms (i.e. their location, as well as, their existence) would be beneficial in identifying disease loci for many disorders, including proliferative diseases and conditions. Such a method is described in US Patents 5,858,659 and 5,856,104. The US Patents referenced supra are hereby incorporated by reference in their entirety herein.

The present invention encompasses polynucleotides of the present invention that are chemically synthesized, or reproduced as peptide nucleic acids (PNA), or according to other methods known in the art. The use of PNAs would serve as the preferred form if the polynucleotides are incorporated onto a solid support, or gene chip. For the purposes of the present invention, a peptide nucleic acid (PNA) is a polyamide type of DNA analog and the monomeric units for adenine, guanine, thymine and cytosine are available commercially (Perceptive Biosystems). Certain components of DNA, such as phosphorus, phosphorus oxides, or deoxyribose derivatives, are not present in PNAs. As disclosed by P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt, Science 254, 1497 (1991); and M. Egholm, O. Buchardt, L.Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden, and P. E. Nielsen, Nature 365, 666 (1993), PNAs bind specifically and

tightly to complementary DNA strands and are not degraded by nucleases. In fact, PNA binds more strongly to DNA than DNA itself does. This is probably because there is no electrostatic repulsion between the two strands, and also the polyamide backbone is more flexible. Because of this, PNA/DNA duplexes bind under a wider  
5 range of stringency conditions than DNA/DNA duplexes, making it easier to perform multiplex hybridization. Smaller probes can be used than with DNA due to the stronger binding characteristics of PNA:DNA hybrids. In addition, it is more likely that single base mismatches can be determined with PNA/DNA hybridization because a single mismatch in a PNA/DNA 15-mer lowers the melting point ( $T_{sub.m}$ ) by 8°-  
10 20° C, vs. 4°-16° C for the DNA/DNA 15-mer duplex. Also, the absence of charge groups in PNA means that hybridization can be done at low ionic strengths and reduce possible interference by salt during the analysis.

In addition to the foregoing, a polynucleotide can be used to control gene expression through triple helix formation or antisense DNA or RNA. Antisense  
15 techniques are discussed, for example, in Okano, J. *Neurochem.* 56: 560 (1991); "Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance Lee et al., *Nucleic Acids Research* 6: 3073 (1979); Cooney et al., *Science* 241: 456 (1988); and Dervan et al., *Science* 251: 1360 (1991). Both methods rely on binding of the  
20 polynucleotide to a complementary DNA or RNA. For these techniques, preferred polynucleotides are usually oligonucleotides 20 to 40 bases in length and complementary to either the region of the gene involved in transcription (triple helix - see Lee et al., *Nucl. Acids Res.* 6:3073 (1979); Cooney et al., *Science* 241:456 (1988); and Dervan et al., *Science* 251:1360 (1991) ) or to the mRNA itself (antisense -  
25 Okano, J. *Neurochem.* 56:560 (1991); Oligodeoxy-nucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988).) Triple helix formation optimally results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Both techniques are effective in model systems, and the information disclosed herein can  
30 be used to design antisense or triple helix polynucleotides in an effort to treat or prevent disease.

The present invention encompasses the addition of a nuclear localization signal, operably linked to the 5' end, 3' end, or any location therein, to any of the oligonucleotides, antisense oligonucleotides, triple helix oligonucleotides, ribozymes, PNA oligonucleotides, and/or polynucleotides, of the present invention. See, for  
5 example, G. Cutrona, et al., Nat. Biotech., 18:300-303, (2000); which is hereby incorporated herein by reference.

Polynucleotides of the present invention are also useful in gene therapy. One goal of gene therapy is to insert a normal gene into an organism having a defective gene, in an effort to correct the genetic defect. The polynucleotides disclosed in the  
10 present invention offer a means of targeting such genetic defects in a highly accurate manner. Another goal is to insert a new gene that was not present in the host genome, thereby producing a new trait in the host cell. In one example, polynucleotide sequences of the present invention may be used to construct chimeric RNA/DNA oligonucleotides corresponding to said sequences, specifically designed to induce host  
15 cell mismatch repair mechanisms in an organism upon systemic injection, for example (Bartlett, R.J., et al., Nat. Biotech, 18:615-622 (2000), which is hereby incorporated by reference herein in its entirety). Such RNA/DNA oligonucleotides could be designed to correct genetic defects in certain host strains, and/or to introduce desired phenotypes in the host (e.g., introduction of a specific polymorphism within an  
20 endogenous gene corresponding to a polynucleotide of the present invention that may ameliorate and/or prevent a disease symptom and/or disorder, etc.). Alternatively, the polynucleotide sequence of the present invention may be used to construct duplex oligonucleotides corresponding to said sequence, specifically designed to correct genetic defects in certain host strains, and/or to introduce desired phenotypes into the  
25 host (e.g., introduction of a specific polymorphism within an endogenous gene corresponding to a polynucleotide of the present invention that may ameliorate and/or prevent a disease symptom and/or disorder, etc.). Such methods of using duplex oligonucleotides are known in the art and are encompassed by the present invention (see EP1007712, which is hereby incorporated by reference herein in its entirety).

30 The polynucleotides are also useful for identifying organisms from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel.

In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identifying personnel. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification  
5 difficult. The polynucleotides of the present invention can be used as additional DNA markers for RFLP.

The polynucleotides of the present invention can also be used as an alternative to RFLP, by determining the actual base-by-base DNA sequence of selected portions of an organisms genome. These sequences can be used to prepare PCR primers for  
10 amplifying and isolating such selected DNA, which can then be sequenced. Using this technique, organisms can be identified because each organism will have a unique set of DNA sequences. Once an unique ID database is established for an organism, positive identification of that organism, living or dead, can be made from extremely small tissue samples. Similarly, polynucleotides of the present invention can be used  
15 as polymorphic markers, in addition to, the identification of transformed or non-transformed cells and/or tissues.

There is also a need for reagents capable of identifying the source of a particular tissue. Such need arises, for example, when presented with tissue of unknown origin. Appropriate reagents can comprise, for example, DNA probes or  
20 primers specific to particular tissue prepared from the sequences of the present invention. Panels of such reagents can identify tissue by species and/or by organ type. In a similar fashion, these reagents can be used to screen tissue cultures for contamination. Moreover, as mentioned above, such reagents can be used to screen and/or identify transformed and non-transformed cells and/or tissues.

In the very least, the polynucleotides of the present invention can be used as  
25 molecular weight markers on Southern gels, as diagnostic probes for the presence of a specific mRNA in a particular cell type, as a probe to "subtract-out" known sequences in the process of discovering novel polynucleotides, for selecting and making oligomers for attachment to a "gene chip" or other support, to raise anti-DNA  
30 antibodies using DNA immunization techniques, and as an antigen to elicit an immune response.

## Uses of the Polypeptides

Each of the polypeptides identified herein can be used in numerous ways. The following description should be considered exemplary and utilizes known techniques.

5 A polypeptide of the present invention can be used to assay protein levels in a biological sample using antibody-based techniques. For example, protein expression in tissues can be studied with classical immunohistological methods. (Jalkanen, M., et al., J. Cell. Biol. 101:976-985 (1985); Jalkanen, M., et al., J. Cell . Biol. 105:3087-3096 (1987).) Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay  
10 (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine ( $^{125}\text{I}$ ,  $^{121}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{112}\text{In}$ ), and technetium ( $^{99\text{m}}\text{Tc}$ ), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

15 In addition to assaying protein levels in a biological sample, proteins can also be detected in vivo by imaging. Antibody labels or markers for in vivo imaging of protein include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and  
20 ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma.

A protein-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example,  $^{131}\text{I}$ ,  $^{112}\text{In}$ ,  $^{99\text{m}}\text{Tc}$ ), a radio-opaque substance, or a material detectable by nuclear  
25 magnetic resonance, is introduced (for example, parenterally, subcutaneously, or intraperitoneally) into the mammal. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5  
30 to 20 millicuries of  $^{99\text{m}}\text{Tc}$ . The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain the specific protein. In vivo tumor imaging is described in S.W. Burchiel et al., "Immunopharmacokinetics of

Radiolabeled Antibodies and Their Fragments." (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982).)

Thus, the invention provides a diagnostic method of a disorder, which  
5 involves (a) assaying the expression of a polypeptide of the present invention in cells or body fluid of an individual; (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of a disorder. With respect to cancer, the presence of a relatively high  
10 amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the  
15 development or further progression of the cancer.

Moreover, polypeptides of the present invention can be used to treat, prevent, and/or diagnose disease. For example, patients can be administered a polypeptide of the present invention in an effort to replace absent or decreased levels of the polypeptide (e.g., insulin), to supplement absent or decreased levels of a different  
20 polypeptide (e.g., hemoglobin S for hemoglobin B, SOD, catalase, DNA repair proteins), to inhibit the activity of a polypeptide (e.g., an oncogene or tumor suppressor), to activate the activity of a polypeptide (e.g., by binding to a receptor), to reduce the activity of a membrane bound receptor by competing with it for free ligand (e.g., soluble TNF receptors used in reducing inflammation), or to bring about a  
25 desired response (e.g., blood vessel growth inhibition, enhancement of the immune response to proliferative cells or tissues).

Similarly, antibodies directed to a polypeptide of the present invention can also be used to treat, prevent, and/or diagnose disease. For example, administration of an antibody directed to a polypeptide of the present invention can bind and reduce  
30 overproduction of the polypeptide. Similarly, administration of an antibody can activate the polypeptide, such as by binding to a polypeptide bound to a membrane (receptor).

At the very least, the polypeptides of the present invention can be used as molecular weight markers on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art. Polypeptides can also be used to raise antibodies, which in turn are used to measure protein expression from a recombinant cell, as a way of assessing transformation of the host cell. Moreover, the polypeptides of the present invention can be used to test the following biological activities.

#### Gene Therapy Methods

Another aspect of the present invention is to gene therapy methods for treating or preventing disorders, diseases and conditions. The gene therapy methods relate to the introduction of nucleic acid (DNA, RNA and antisense DNA or RNA) sequences into an animal to achieve expression of a polypeptide of the present invention. This method requires a polynucleotide which codes for a polypeptide of the invention that operatively linked to a promoter and any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques are known in the art, see, for example, WO90/11092, which is herein incorporated by reference.

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) comprising a promoter operably linked to a polynucleotide of the invention ex vivo, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, see Belldgrun et al., J. Natl. Cancer Inst., 85:207-216 (1993); Ferrantini et al., Cancer Research, 53:107-1112 (1993); Ferrantini et al., J. Immunology 153: 4604-4615 (1994); Kaido, T., et al., Int. J. Cancer 60: 221-229 (1995); Ogura et al., Cancer Research 50: 5102-5106 (1990); Santodonato, et al., Human Gene Therapy 7:1-10 (1996); Santodonato, et al., Gene Therapy 4:1246-1255 (1997); and Zhang, et al., Cancer Gene Therapy 3: 31-38 (1996)), which are herein incorporated by reference. In one embodiment, the cells which are engineered are arterial cells. The arterial cells may be reintroduced into the patient through direct injection to the artery, the tissues surrounding the artery, or through catheter injection.

As discussed in more detail below, the polynucleotide constructs can be delivered by any method that delivers injectable materials to the cells of an animal,



such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, and the like). The polynucleotide constructs may be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

In one embodiment, the polynucleotide of the invention is delivered as a naked  
5 polynucleotide. The term "naked" polynucleotide, DNA or RNA refers to sequences that are free from any delivery vehicle that acts to assist, promote or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the polynucleotides of the invention can also be delivered in liposome formulations and lipofectin formulations  
10 and the like can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Patent Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference.

The polynucleotide vector constructs of the invention used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will  
15 they contain sequences that allow for replication. Appropriate vectors include pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; pSVK3, pBPV, pMSG and pSVL available from Pharmacia; and pEF1/V5, pcDNA3.1, and pRc/CMV2 available from Invitrogen. Other suitable vectors will be readily apparent to the skilled artisan.

20 Any strong promoter known to those skilled in the art can be used for driving the expression of polynucleotide sequence of the invention. Suitable promoters include adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT  
25 promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs; the b-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter for the polynucleotides of the invention.

30 Unlike other gene therapy techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA

sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The polynucleotide construct of the invention can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. In vivo muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked nucleic acid sequence injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 mg/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration.

The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked DNA

constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The naked polynucleotides are delivered by any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous  
5 injection, topical administration, catheter infusion, and so-called "gene guns". These delivery methods are known in the art.

The constructs may also be delivered with delivery vehicles such as viral sequences, viral particles, liposome formulations, lipofectin, precipitating agents, etc. Such methods of delivery are known in the art.

10 In certain embodiments, the polynucleotide constructs of the invention are complexed in a liposome preparation. Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. However, cationic liposomes are particularly preferred because a tight charge complex can be formed between the cationic liposome and the  
15 polyanionic nucleic acid. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., Proc. Natl. Acad. Sci. USA , 84:7413-7416 (1987), which is herein incorporated by reference); mRNA (Malone et al., Proc. Natl. Acad. Sci. USA , 86:6077-6081 (1989), which is herein incorporated by reference); and purified transcription factors (Debs et al., J. Biol. Chem.,  
20 265:10189-10192 (1990), which is herein incorporated by reference), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are particularly useful and are available under the trademark Lipofectin, from GIBCO BRL, Grand  
25 Island, N.Y. (See, also, Felgner et al., Proc. Natl. Acad. Sci. USA , 84:7413-7416 (1987), which is herein incorporated by reference). Other commercially available liposomes include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boehringer).

Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g. PCT Publication NO: WO 90/11092  
30 (which is herein incorporated by reference) for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. Preparation of DOTMA liposomes is explained in the literature, see, e.g., Felgner et al., Proc.

Natl. Acad. Sci. USA, 84:7413-7417, which is herein incorporated by reference. Similar methods can be used to prepare liposomes from other cationic lipid materials.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, Ala.), or can be easily prepared using readily available materials. Such materials include phosphatidyl, choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphosphatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

For example, commercially dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), and dioleoylphosphatidyl ethanolamine (DOPE) can be used in various combinations to make conventional liposomes, with or without the addition of cholesterol. Thus, for example, DOPG/DOPC vesicles can be prepared by drying 50 mg each of DOPG and DOPC under a stream of nitrogen gas into a sonication vial. The sample is placed under a vacuum pump overnight and is hydrated the following day with deionized water. The sample is then sonicated for 2 hours in a capped vial, using a Heat Systems model 350 sonicator equipped with an inverted cup (bath type) probe at the maximum setting while the bath is circulated at 15EC. Alternatively, negatively charged vesicles can be prepared without sonication to produce multilamellar vesicles or by extrusion through nucleopore membranes to produce unilamellar vesicles of discrete size. Other methods are known and available to those of skill in the art.

The liposomes can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs), with SUVs being preferred. The various liposome-nucleic acid complexes are prepared using methods well known in the art. See, e.g., Straubinger et al., *Methods of Immunology*, 101:512-527 (1983), which is herein incorporated by reference. For example, MLVs containing nucleic acid can be prepared by depositing a thin film of phospholipid on the walls of a glass tube and subsequently hydrating with a solution of the material to be encapsulated. SUVs are prepared by extended sonication of MLVs to produce a homogeneous population of unilamellar liposomes. The material to be entrapped is added to a

suspension of preformed MLVs and then sonicated. When using liposomes containing cationic lipids, the dried lipid film is resuspended in an appropriate solution such as sterile water or an isotonic buffer solution such as 10 mM Tris/NaCl, sonicated, and then the preformed liposomes are mixed directly with the DNA. The liposome and DNA form a very stable complex due to binding of the positively charged liposomes to the cationic DNA. SUVs find use with small nucleic acid fragments. LUVs are prepared by a number of methods, well known in the art. Commonly used methods include Ca<sup>2+</sup>-EDTA chelation (Papahadjopoulos et al., *Biochim. Biophys. Acta*, 394:483 (1975); Wilson et al., *Cell*, 17:77 (1979)); ether injection (Deamer et al., *Biochim. Biophys. Acta*, 443:629 (1976); Ostro et al., *Biochem. Biophys. Res. Commun.*, 76:836 (1977); Fraley et al., *Proc. Natl. Acad. Sci. USA*, 76:3348 (1979)); detergent dialysis (Enoch et al., *Proc. Natl. Acad. Sci. USA*, 76:145 (1979)); and reverse-phase evaporation (REV) (Fraley et al., *J. Biol. Chem.*, 255:10431 (1980); Szoka et al., *Proc. Natl. Acad. Sci. USA*, 75:145 (1978); Schaefer-Ridder et al., *Science*, 215:166 (1982)), which are herein incorporated by reference.

Generally, the ratio of DNA to liposomes will be from about 10:1 to about 1:10. Preferably, the ration will be from about 5:1 to about 1:5. More preferably, the ration will be about 3:1 to about 1:3. Still more preferably, the ratio will be about 1:1.

U.S. Patent NO: 5,676,954 (which is herein incorporated by reference) reports on the injection of genetic material, complexed with cationic liposomes carriers, into mice. U.S. Patent Nos. 4,897,355, 4,946,787, 5,049,386, 5,459,127, 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication NO: WO 94/9469 (which are herein incorporated by reference) provide cationic lipids for use in transfecting DNA into cells and mammals. U.S. Patent Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication NO: WO 94/9469 (which are herein incorporated by reference) provide methods for delivering DNA-cationic lipid complexes to mammals.

In certain embodiments, cells are engineered, ex vivo or in vivo, using a retroviral particle containing RNA which comprises a sequence encoding polypeptides of the invention. Retroviruses from which the retroviral plasmid vectors may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, Rous sarcoma Virus, Harvey Sarcoma Virus, avian leukosis

virus, gibbon ape leukemia virus, human immunodeficiency virus, Myeloproliferative Sarcoma Virus, and mammary tumor virus.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, R-2, R-AM, PA12, T19-14X, VT-19-17-H2, RCRE, RCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy , 1:5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO<sub>4</sub> precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include polynucleotide encoding polypeptides of the invention. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express polypeptides of the invention.

In certain other embodiments, cells are engineered, ex vivo or in vivo, with polynucleotides of the invention contained in an adenovirus vector. Adenovirus can be manipulated such that it encodes and expresses polypeptides of the invention, and at the same time is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. Adenovirus expression is achieved without integration of the viral DNA into the host cell chromosome, thereby alleviating concerns about insertional mutagenesis. Furthermore, adenoviruses have been used as live enteric vaccines for many years with an excellent safety profile (Schwartz et al., Am. Rev. Respir. Dis., 109:233-238 (1974)). Finally, adenovirus mediated gene transfer has been demonstrated in a number of instances including transfer of alpha-1-antitrypsin and CFTR to the lungs of cotton rats (Rosenfeld et al., Science, 252:431-434 (1991); Rosenfeld et al., Cell, 68:143-155 (1992)). Furthermore, extensive studies to attempt to establish adenovirus as a causative agent in human cancer were uniformly negative (Green et al. Proc. Natl. Acad. Sci. USA , 76:6606 (1979)).

Suitable adenoviral vectors useful in the present invention are described, for example, in Kozarsky and Wilson, Curr. Opin. Genet. Devel., 3:499-503 (1993);

Rosenfeld et al., *Cell*, 68:143-155 (1992); Engelhardt et al., *Human Genet. Ther.*, 4:759-769 (1993); Yang et al., *Nature Genet.*, 7:362-369 (1994); Wilson et al., *Nature*, 365:691-692 (1993); and U.S. Patent NO: 5,652,224, which are herein incorporated by reference. For example, the adenovirus vector Ad2 is useful and can be grown in human 293 cells. These cells contain the E1 region of adenovirus and constitutively express E1a and E1b, which complement the defective adenoviruses by providing the products of the genes deleted from the vector. In addition to Ad2, other varieties of adenovirus (e.g., Ad3, Ad5, and Ad7) are also useful in the present invention.

10        Preferably, the adenoviruses used in the present invention are replication deficient. Replication deficient adenoviruses require the aid of a helper virus and/or packaging cell line to form infectious particles. The resulting virus is capable of infecting cells and can express a polynucleotide of interest which is operably linked to a promoter, but cannot replicate in most cells. Replication deficient adenoviruses may be deleted in one or more of all or a portion of the following genes: E1a, E1b, E3, E4, E2a, or L1 through L5.

      In certain other embodiments, the cells are engineered, ex vivo or in vivo, using an adeno-associated virus (AAV). AAVs are naturally occurring defective viruses that require helper viruses to produce infectious particles (Muzyczka, *Curr. Topics in Microbiol. Immunol.*, 158:97 (1992)). It is also one of the few viruses that may integrate its DNA into non-dividing cells. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate, but space for exogenous DNA is limited to about 4.5 kb. Methods for producing and using such AAVs are known in the art. See, for example, U.S. Patent Nos. 5,139,941, 5,173,414, 5,354,678, 5,436,146, 5,474,935, 5,478,745, and 5,589,377.

      For example, an appropriate AAV vector for use in the present invention will include all the sequences necessary for DNA replication, encapsidation, and host-cell integration. The polynucleotide construct containing polynucleotides of the invention is inserted into the AAV vector using standard cloning methods, such as those found in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (1989). The recombinant AAV vector is then transfected into packaging cells which are infected with a helper virus, using any standard technique, including

lipofection, electroporation, calcium phosphate precipitation, etc. Appropriate helper viruses include adenoviruses, cytomegaloviruses, vaccinia viruses, or herpes viruses. Once the packaging cells are transfected and infected, they will produce infectious AAV viral particles which contain the polynucleotide construct of the invention.

5 These viral particles are then used to transduce eukaryotic cells, either ex vivo or in vivo. The transduced cells will contain the polynucleotide construct integrated into its genome, and will express the desired gene product.

Another method of gene therapy involves operably associating heterologous control regions and endogenous polynucleotide sequences (e.g. encoding the

10 polypeptide sequence of interest) via homologous recombination (see, e.g., U.S. Patent NO: 5,641,670, issued June 24, 1997; International Publication NO: WO 96/29411, published September 26, 1996; International Publication NO: WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA, 86:8932-8935 (1989); and Zijlstra et al., Nature, 342:435-438 (1989). This method

15 involves the activation of a gene which is present in the target cells, but which is not normally expressed in the cells, or is expressed at a lower level than desired.

Polynucleotide constructs are made, using standard techniques known in the art, which contain the promoter with targeting sequences flanking the promoter. Suitable promoters are described herein. The targeting sequence is sufficiently

20 complementary to an endogenous sequence to permit homologous recombination of the promoter-targeting sequence with the endogenous sequence. The targeting sequence will be sufficiently near the 5' end of the desired endogenous polynucleotide sequence so the promoter will be operably linked to the endogenous sequence upon homologous recombination.

25 The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the

30 amplified promoter. The amplified promoter and targeting sequences are digested and ligated together.



The promoter-targeting sequence construct is delivered to the cells, either as naked polynucleotide, or in conjunction with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, whole viruses, lipofection, precipitating agents, etc., described in more detail above. The P promoter-targeting sequence can  
5 be delivered by any method, included direct needle injection, intravenous injection, topical administration, catheter infusion, particle accelerators, etc. The methods are described in more detail below.

The promoter-targeting sequence construct is taken up by cells. Homologous recombination between the construct and the endogenous sequence takes place, such  
10 that an endogenous sequence is placed under the control of the promoter. The promoter then drives the expression of the endogenous sequence.

The polynucleotides encoding polypeptides of the present invention may be administered along with other polynucleotides encoding angiogenic proteins. Angiogenic proteins include, but are not limited to, acidic and basic fibroblast tubulin  
15 tyrosine ligase proteins, VEGF-1, VEGF-2 (VEGF-C), VEGF-3 (VEGF-B), epidermal tubulin tyrosine ligase protein alpha and beta, platelet-derived endothelial cell tubulin tyrosine ligase protein, platelet-derived tubulin tyrosine ligase protein, tumor necrosis factor alpha, hepatocyte tubulin tyrosine ligase protein, insulin like  
20 tubulin tyrosine ligase protein, colony stimulating factor, macrophage colony stimulating factor, granulocyte/macrophage colony stimulating factor, and nitric oxide synthase.

Preferably, the polynucleotide encoding a polypeptide of the invention contains a secretory signal sequence that facilitates secretion of the protein. Typically, the signal sequence is positioned in the coding region of the polynucleotide to be  
25 expressed towards or at the 5' end of the coding region. The signal sequence may be homologous or heterologous to the polynucleotide of interest and may be homologous or heterologous to the cells to be transfected. Additionally, the signal sequence may be chemically synthesized using methods known in the art.

Any mode of administration of any of the above-described polynucleotides  
30 constructs can be used so long as the mode results in the expression of one or more molecules in an amount sufficient to provide a therapeutic effect. This includes direct needle injection, systemic injection, catheter infusion, biolistic injectors, particle

accelerators (i.e., "gene guns"), gelfoam sponge depots, other commercially available depot materials, osmotic pumps (e.g., Alza minipumps), oral or suppository solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. For example, direct injection of naked calcium phosphate-precipitated plasmid into rat liver and rat spleen or a protein-coated plasmid into the portal vein  
5 has resulted in gene expression of the foreign gene in the rat livers. (Kaneda et al., Science, 243:375 (1989)).

A preferred method of local administration is by direct injection. Preferably, a recombinant molecule of the present invention complexed with a delivery vehicle is  
10 administered by direct injection into or locally within the area of arteries. Administration of a composition locally within the area of arteries refers to injecting the composition centimeters and preferably, millimeters within arteries.

Another method of local administration is to contact a polynucleotide construct of the present invention in or around a surgical wound. For example, a  
15 patient can undergo surgery and the polynucleotide construct can be coated on the surface of tissue inside the wound or the construct can be injected into areas of tissue inside the wound.

Therapeutic compositions useful in systemic administration, include recombinant molecules of the present invention complexed to a targeted delivery  
20 vehicle of the present invention. Suitable delivery vehicles for use with systemic administration comprise liposomes comprising ligands for targeting the vehicle to a particular site.

Preferred methods of systemic administration, include intravenous injection, aerosol, oral and percutaneous (topical) delivery. Intravenous injections can be  
25 performed using methods standard in the art. Aerosol delivery can also be performed using methods standard in the art (see, for example, Stribling et al., Proc. Natl. Acad. Sci. USA , 189:11277-11281 (1992), which is incorporated herein by reference). Oral delivery can be performed by complexing a polynucleotide construct of the present invention to a carrier capable of withstanding degradation by digestive enzymes in the  
30 gut of an animal. Examples of such carriers, include plastic capsules or tablets, such as those known in the art. Topical delivery can be performed by mixing a

polynucleotide construct of the present invention with a lipophilic reagent (e.g., DMSO) that is capable of passing into the skin.

Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the animal, the precise condition requiring treatment and its severity, and the route of administration. The frequency of treatments depends upon a number of factors, such as the amount of polynucleotide constructs administered per dose, as well as the health and history of the subject. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian. Therapeutic compositions of the present invention can be administered to any animal, preferably to mammals and birds. Preferred mammals include humans, dogs, cats, mice, rats, rabbits sheep, cattle, horses and pigs, with humans being particularly preferred.

#### Biological Activities

The polynucleotides or polypeptides, or agonists or antagonists of the present invention can be used in assays to test for one or more biological activities. If these polynucleotides and polypeptides do exhibit activity in a particular assay, it is likely that these molecules may be involved in the diseases associated with the biological activity. Thus, the polynucleotides or polypeptides, or agonists or antagonists could be used to treat the associated disease.

#### Immune Activity

The polynucleotides or polypeptides, or agonists or antagonists of the present invention may be useful in treating, preventing, and/or diagnosing diseases, disorders, and/or conditions of the immune system, by activating or inhibiting the proliferation, differentiation, or mobilization (chemotaxis) of immune cells. Immune cells develop through a process called hematopoiesis, producing myeloid (platelets, red blood cells, neutrophils, and macrophages) and lymphoid (B and T lymphocytes) cells from pluripotent stem cells. The etiology of these immune diseases, disorders, and/or conditions may be genetic, somatic, such as cancer or some autoimmune diseases, disorders, and/or conditions, acquired (e.g., by chemotherapy or toxins), or infectious. Moreover, a polynucleotides or polypeptides, or agonists or antagonists of the present

invention can be used as a marker or detector of a particular immune system disease or disorder.

A polynucleotides or polypeptides, or agonists or antagonists of the present invention may be useful in treating, preventing, and/or diagnosing diseases, disorders, and/or conditions of hematopoietic cells. A polynucleotides or polypeptides, or agonists or antagonists of the present invention could be used to increase differentiation and proliferation of hematopoietic cells, including the pluripotent stem cells, in an effort to treat or prevent those diseases, disorders, and/or conditions associated with a decrease in certain (or many) types hematopoietic cells. Examples of immunologic deficiency syndromes include, but are not limited to: blood protein diseases, disorders, and/or conditions (e.g. agammaglobulinemia, dysgammaglobulinemia), ataxia telangiectasia, common variable immunodeficiency, Digeorge Syndrome, HIV infection, HTLV-BLV infection, leukocyte adhesion deficiency syndrome, lymphopenia, phagocyte bactericidal dysfunction, severe combined immunodeficiency (SCIDs), Wiskott-Aldrich Disorder, anemia, thrombocytopenia, or hemoglobinuria.

Moreover, a polynucleotides or polypeptides, or agonists or antagonists of the present invention could also be used to modulate hemostatic (the stopping of bleeding) or thrombolytic activity (clot formation). For example, by increasing hemostatic or thrombolytic activity, a polynucleotides or polypeptides, or agonists or antagonists of the present invention could be used to treat or prevent blood coagulation diseases, disorders, and/or conditions (e.g., afibrinogenemia, factor deficiencies, arterial thrombosis, venous thrombosis, etc.), blood platelet diseases, disorders, and/or conditions (e.g. thrombocytopenia), or wounds resulting from trauma, surgery, or other causes. Alternatively, a polynucleotides or polypeptides, or agonists or antagonists of the present invention that can decrease hemostatic or thrombolytic activity could be used to inhibit or dissolve clotting. Polynucleotides or polypeptides, or agonists or antagonists of the present invention are may also be useful for the detection, prognosis, treatment, and/or prevention of heart attacks (infarction), strokes, scarring, fibrinolysis, uncontrolled bleeding, uncontrolled coagulation, uncontrolled complement fixation, and/or inflammation.

A polynucleotides or polypeptides, or agonists or antagonists of the present invention may also be useful in treating, preventing, and/or diagnosing autoimmune diseases, disorders, and/or conditions. Many autoimmune diseases, disorders, and/or conditions result from inappropriate recognition of self as foreign material by immune cells. This inappropriate recognition results in an immune response leading to the destruction of the host tissue. Therefore, the administration of a polynucleotides or polypeptides, or agonists or antagonists of the present invention that inhibits an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing autoimmune diseases, disorders, and/or conditions.

Examples of autoimmune diseases, disorders, and/or conditions that can be treated, prevented, and/or diagnosed or detected by the present invention include, but are not limited to: Addison's Disease, hemolytic anemia, antiphospholipid syndrome, rheumatoid arthritis, dermatitis, allergic encephalomyelitis, glomerulonephritis, Goodpasture's Syndrome, Graves' Disease, Multiple Sclerosis, Myasthenia Gravis, Neuritis, Ophthalmia, Bullous Pemphigoid, Pemphigus, Polyendocrinopathies, Purpura, Reiter's Disease, Stiff-Man Syndrome, Autoimmune Thyroiditis, Systemic Lupus Erythematosus, Autoimmune Pulmonary Inflammation, Guillain-Barre Syndrome, insulin dependent diabetes mellitis, and autoimmune inflammatory eye disease.

Similarly, allergic reactions and conditions, such as asthma (particularly allergic asthma) or other respiratory problems, may also be treated, prevented, and/or diagnosed by polynucleotides or polypeptides, or agonists or antagonists of the present invention. Moreover, these molecules can be used to treat anaphylaxis, hypersensitivity to an antigenic molecule, or blood group incompatibility.

A polynucleotides or polypeptides, or agonists or antagonists of the present invention may also be used to treat, prevent, and/or diagnose organ rejection or graft-versus-host disease (GVHD). Organ rejection occurs by host immune cell destruction of the transplanted tissue through an immune response. Similarly, an immune response is also involved in GVHD, but, in this case, the foreign transplanted immune cells destroy the host tissues. The administration of a polynucleotides or polypeptides, or agonists or antagonists of the present invention that inhibits an immune response,

particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing organ rejection or GVHD.

Similarly, a polynucleotides or polypeptides, or agonists or antagonists of the present invention may also be used to modulate inflammation. For example, the polypeptide or polynucleotide or agonists or antagonist may inhibit the proliferation and differentiation of cells involved in an inflammatory response. These molecules can be used to treat, prevent, and/or diagnose inflammatory conditions, both chronic and acute conditions, including chronic prostatitis, granulomatous prostatitis and malacoplakia, inflammation associated with infection (e.g., septic shock, sepsis, or systemic inflammatory response syndrome (SIRS)), ischemia-reperfusion injury, endotoxin lethality, arthritis, complement-mediated hyperacute rejection, nephritis, cytokine or chemokine induced lung injury, inflammatory bowel disease, Crohn's disease, or resulting from over production of cytokines (e.g., TNF or IL-1.)

#### Hyperproliferative Disorders

A polynucleotides or polypeptides, or agonists or antagonists of the invention can be used to treat, prevent, and/or diagnose hyperproliferative diseases, disorders, and/or conditions, including neoplasms. A polynucleotides or polypeptides, or agonists or antagonists of the present invention may inhibit the proliferation of the disorder through direct or indirect interactions. Alternatively, a polynucleotides or polypeptides, or agonists or antagonists of the present invention may proliferate other cells which can inhibit the hyperproliferative disorder.

For example, by increasing an immune response, particularly increasing antigenic qualities of the hyperproliferative disorder or by proliferating, differentiating, or mobilizing T-cells, hyperproliferative diseases, disorders, and/or conditions can be treated, prevented, and/or diagnosed. This immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, decreasing an immune response may also be a method of treating, preventing, and/or diagnosing hyperproliferative diseases, disorders, and/or conditions, such as a chemotherapeutic agent.

Examples of hyperproliferative diseases, disorders, and/or conditions that can be treated, prevented, and/or diagnosed by polynucleotides or polypeptides, or agonists or antagonists of the present invention include, but are not limited to

neoplasms located in the: colon, abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic, and urogenital.

5           Similarly, other hyperproliferative diseases, disorders, and/or conditions can also be treated, prevented, and/or diagnosed by a polynucleotides or polypeptides, or agonists or antagonists of the present invention. Examples of such hyperproliferative diseases, disorders, and/or conditions include, but are not limited to: hypergammaglobulinemia, lymphoproliferative diseases, disorders, and/or conditions,  
10       paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenstrom's Macroglobulinemia, Gaucher's Disease, histiocytosis, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

          One preferred embodiment utilizes polynucleotides of the present invention to inhibit aberrant cellular division, by gene therapy using the present invention, and/or  
15       protein fusions or fragments thereof.

          Thus, the present invention provides a method for treating or preventing cell proliferative diseases, disorders, and/or conditions by inserting into an abnormally proliferating cell a polynucleotide of the present invention, wherein said polynucleotide represses said expression.

20           Another embodiment of the present invention provides a method of treating or preventing cell-proliferative diseases, disorders, and/or conditions in individuals comprising administration of one or more active gene copies of the present invention to an abnormally proliferating cell or cells. In a preferred embodiment, polynucleotides of the present invention is a DNA construct comprising a  
25       recombinant expression vector effective in expressing a DNA sequence encoding said polynucleotides. In another preferred embodiment of the present invention, the DNA construct encoding the polynucleotides of the present invention is inserted into cells to be treated utilizing a retrovirus, or more Preferably an adenoviral vector (See G J. Nabel, et. al., PNAS 1999 96: 324-326, which is hereby incorporated by reference). In  
30       a most preferred embodiment, the viral vector is defective and will not transform non-proliferating cells, only proliferating cells. Moreover, in a preferred embodiment, the polynucleotides of the present invention inserted into proliferating cells either alone,

or in combination with or fused to other polynucleotides, can then be modulated via an external stimulus (i.e. magnetic, specific small molecule, chemical, or drug administration, etc.), which acts upon the promoter upstream of said polynucleotides to induce expression of the encoded protein product. As such the beneficial  
5 therapeutic affect of the present invention may be expressly modulated (i.e. to increase, decrease, or inhibit expression of the present invention) based upon said external stimulus.

Polynucleotides of the present invention may be useful in repressing expression of oncogenic genes or antigens. By "repressing expression of the  
10 oncogenic genes " is intended the suppression of the transcription of the gene, the degradation of the gene transcript (pre-message RNA), the inhibition of splicing, the destruction of the messenger RNA, the prevention of the post-translational modifications of the protein, the destruction of the protein, or the inhibition of the normal function of the protein.

15 For local administration to abnormally proliferating cells, polynucleotides of the present invention may be administered by any method known to those of skill in the art including, but not limited to transfection, electroporation, microinjection of cells, or in vehicles such as liposomes, lipofectin, or as naked polynucleotides, or any other method described throughout the specification. The polynucleotide of the  
20 present invention may be delivered by known gene delivery systems such as, but not limited to, retroviral vectors (Gilboa, J. Virology 44:845 (1982); Hocke, Nature 320:275 (1986); Wilson, et al., Proc. Natl. Acad. Sci. U.S.A. 85:3014), vaccinia virus system (Chakrabarty et al., Mol. Cell Biol. 5:3403 (1985) or other efficient DNA delivery systems (Yates et al., Nature 313:812 (1985)) known to those skilled in the  
25 art. These references are exemplary only and are hereby incorporated by reference. In order to specifically deliver or transfect cells which are abnormally proliferating and spare non-dividing cells, it is preferable to utilize a retrovirus, or adenoviral (as described in the art and elsewhere herein) delivery system known to those of skill in the art. Since host DNA replication is required for retroviral DNA to integrate and the  
30 retrovirus will be unable to self replicate due to the lack of the retrovirus genes needed for its life cycle. Utilizing such a retroviral delivery system for



polynucleotides of the present invention will target said gene and constructs to abnormally proliferating cells and will spare the non-dividing normal cells.

5 The polynucleotides of the present invention may be delivered directly to cell proliferative disorder/disease sites in internal organs, body cavities and the like by use of imaging devices used to guide an injecting needle directly to the disease site. The polynucleotides of the present invention may also be administered to disease sites at the time of surgical intervention.

10 By "cell proliferative disease" is meant any human or animal disease or disorder, affecting any one or any combination of organs, cavities, or body parts, which is characterized by single or multiple local abnormal proliferations of cells, groups of cells, or tissues, whether benign or malignant.

15 Any amount of the polynucleotides of the present invention may be administered as long as it has a biologically inhibiting effect on the proliferation of the treated cells. Moreover, it is possible to administer more than one of the polynucleotide of the present invention simultaneously to the same site. By "biologically inhibiting" is meant partial or total growth inhibition as well as decreases in the rate of proliferation or growth of the cells. The biologically inhibitory dose may be determined by assessing the effects of the polynucleotides of the present invention on target malignant or abnormally proliferating cell growth in tissue culture, tumor growth in animals and cell cultures, or any other method known to one of  
20 ordinary skill in the art.

25 The present invention is further directed to antibody-based therapies which involve administering of anti-polypeptides and anti-polynucleotide antibodies to a mammalian, preferably human, patient for treating, preventing, and/or diagnosing one or more of the described diseases, disorders, and/or conditions. Methods for producing anti-polypeptides and anti-polynucleotide antibodies polyclonal and monoclonal antibodies are described in detail elsewhere herein. Such antibodies may be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

30 A summary of the ways in which the antibodies of the present invention may be used therapeutically includes binding polynucleotides or polypeptides of the present invention locally or systemically in the body or by direct cytotoxicity of the

antibody, e.g. as mediated by complement (CDC) or by effector cells (ADCC). Some of these approaches are described in more detail below. Armed with the teachings provided herein, one of ordinary skill in the art will know how to use the antibodies of the present invention for diagnostic, monitoring or therapeutic purposes without  
5 undue experimentation.

In particular, the antibodies, fragments and derivatives of the present invention are useful for treating, preventing, and/or diagnosing a subject having or developing cell proliferative and/or differentiation diseases, disorders, and/or conditions as described herein. Such treatment comprises administering a single or multiple doses  
10 of the antibody, or a fragment, derivative, or a conjugate thereof.

The antibodies of this invention may be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hematopoietic tubulin tyrosine ligase proteins, for example, which serve to increase the number or activity of effector cells which interact with the antibodies.

It is preferred to use high affinity and/or potent in vivo inhibiting and/or neutralizing antibodies against polypeptides or polynucleotides of the present invention, fragments or regions thereof, for both immunoassays directed to and therapy of diseases, disorders, and/or conditions related to polynucleotides or polypeptides, including fragments thereof, of the present invention. Such antibodies,  
15 fragments, or regions, will preferably have an affinity for polynucleotides or polypeptides, including fragments thereof. Preferred binding affinities include those with a dissociation constant or  $K_d$  less than  $5 \times 10^{-6}M$ ,  $10^{-6}M$ ,  $5 \times 10^{-7}M$ ,  $10^{-7}M$ ,  $5 \times 10^{-8}M$ ,  $10^{-8}M$ ,  $5 \times 10^{-9}M$ ,  $10^{-9}M$ ,  $5 \times 10^{-10}M$ ,  $10^{-10}M$ ,  $5 \times 10^{-11}M$ ,  $10^{-11}M$ ,  $5 \times 10^{-12}M$ ,  $10^{-12}M$ ,  $5 \times 10^{-13}M$ ,  $10^{-13}M$ ,  $5 \times 10^{-14}M$ ,  $10^{-14}M$ ,  $5 \times 10^{-15}M$ , and  $10^{-15}M$ .  
20  
25

Moreover, polypeptides of the present invention may be useful in inhibiting the angiogenesis of proliferative cells or tissues, either alone, as a protein fusion, or in combination with other polypeptides directly or indirectly, as described elsewhere herein. In a most preferred embodiment, said anti-angiogenesis effect may be  
30 achieved indirectly, for example, through the inhibition of hematopoietic, tumor-specific cells, such as tumor-associated macrophages (See Joseph IB, et al. J Natl Cancer Inst, 90(21):1648-53 (1998), which is hereby incorporated by reference).

Antibodies directed to polypeptides or polynucleotides of the present invention may also result in inhibition of angiogenesis directly, or indirectly (See Witte L, et al., Cancer Metastasis Rev. 17(2):155-61 (1998), which is hereby incorporated by reference)).

5 Polypeptides, including protein fusions, of the present invention, or fragments thereof may be useful in inhibiting proliferative cells or tissues through the induction of apoptosis. Said polypeptides may act either directly, or indirectly to induce apoptosis of proliferative cells and tissues, for example in the activation of a death-domain receptor, such as tumor necrosis factor (TNF) receptor-1, CD95 (Fas/APO-1),  
10 TNF-receptor-related apoptosis-mediated protein (TRAMP) and TNF-related apoptosis-inducing ligand (TRAIL) receptor-1 and -2 (See Schulze-Osthoff K, et al., Eur J Biochem 254(3):439-59 (1998), which is hereby incorporated by reference). Moreover, in another preferred embodiment of the present invention, said polypeptides may induce apoptosis through other mechanisms, such as in the  
15 activation of other proteins which will activate apoptosis, or through stimulating the expression of said proteins, either alone or in combination with small molecule drugs or adjuvants, such as apoptonin, galectins, thioredoxins, antiinflammatory proteins (See for example, Mutat. Res. 400(1-2):447-55 (1998), Med Hypotheses.50(5):423-33 (1998), Chem. Biol. Interact. Apr 24;111-112:23-34 (1998), J Mol Med.76(6):402-12  
20 (1998), Int. J. Tissue React. 20(1):3-15 (1998), which are all hereby incorporated by reference).

Polypeptides, including protein fusions to, or fragments thereof, of the present invention are useful in inhibiting the metastasis of proliferative cells or tissues. Inhibition may occur as a direct result of administering polypeptides, or antibodies  
25 directed to said polypeptides as described elsewhere herein, or indirectly, such as activating the expression of proteins known to inhibit metastasis, for example alpha 4 integrins, (See, e.g., Curr Top Microbiol Immunol 1998;231:125-41, which is hereby incorporated by reference). Such therapeutic affects of the present invention may be achieved either alone, or in combination with small molecule drugs or adjuvants.

30 In another embodiment, the invention provides a method of delivering compositions containing the polypeptides of the invention (e.g., compositions containing polypeptides or polypeptide antibodies associated with heterologous

polypeptides, heterologous nucleic acids, toxins, or prodrugs) to targeted cells expressing the polypeptide of the present invention. Polypeptides or polypeptide antibodies of the invention may be associated with heterologous polypeptides, heterologous nucleic acids, toxins, or prodrugs via hydrophobic, hydrophilic, ionic  
5 and/or covalent interactions.

Polypeptides, protein fusions to, or fragments thereof, of the present invention are useful in enhancing the immunogenicity and/or antigenicity of proliferating cells or tissues, either directly, such as would occur if the polypeptides of the present invention 'vaccinated' the immune response to respond to proliferative antigens and  
10 immunogens, or indirectly, such as in activating the expression of proteins known to enhance the immune response (e.g. chemokines), to said antigens and immunogens.

#### Cardiovascular Disorders

Polynucleotides or polypeptides, or agonists or antagonists of the invention may be used to treat, prevent, and/or diagnose cardiovascular diseases, disorders,  
15 and/or conditions, including peripheral artery disease, such as limb ischemia.

Cardiovascular diseases, disorders, and/or conditions include cardiovascular abnormalities, such as arterio-arterial fistula, arteriovenous fistula, cerebral arteriovenous malformations, congenital heart defects, pulmonary atresia, and Scimitar Syndrome. Congenital heart defects include aortic coarctation, cor  
20 triatriatum, coronary vessel anomalies, crisscross heart, dextrocardia, patent ductus arteriosus, Ebstein's anomaly, Eisenmenger complex, hypoplastic left heart syndrome, levocardia, tetralogy of fallot, transposition of great vessels, double outlet right ventricle, tricuspid atresia, persistent truncus arteriosus, and heart septal defects, such as aortopulmonary septal defect, endocardial cushion defects, Lutembacher's  
25 Syndrome, trilogy of Fallot, ventricular heart septal defects.

Cardiovascular diseases, disorders, and/or conditions also include heart disease, such as arrhythmias, carcinoid heart disease, high cardiac output, low cardiac output, cardiac tamponade, endocarditis (including bacterial), heart aneurysm, cardiac arrest, congestive heart failure, congestive cardiomyopathy, paroxysmal dyspnea,  
30 cardiac edema, heart hypertrophy, congestive cardiomyopathy, left ventricular hypertrophy, right ventricular hypertrophy, post-infarction heart rupture, ventricular septal rupture, heart valve diseases, myocardial diseases, myocardial ischemia,

pericardial effusion, pericarditis (including constrictive and tuberculous), pneumopericardium, postpericardiotomy syndrome, pulmonary heart disease, rheumatic heart disease, ventricular dysfunction, hyperemia, cardiovascular pregnancy complications, Scimitar Syndrome, cardiovascular syphilis, and  
 5 cardiovascular tuberculosis.

Arrhythmias include sinus arrhythmia, atrial fibrillation, atrial flutter, bradycardia, extrasystole, Adams-Stokes Syndrome, bundle-branch block, sinoatrial block, long QT syndrome, parasystole, Lown-Ganong-Levine Syndrome, Mahaim-type pre-excitation syndrome, Wolff-Parkinson-White syndrome, sick sinus  
 10 syndrome, tachycardias, and ventricular fibrillation. Tachycardias include paroxysmal tachycardia, supraventricular tachycardia, accelerated idioventricular rhythm, atrioventricular nodal reentry tachycardia, ectopic atrial tachycardia, ectopic junctional tachycardia, sinoatrial nodal reentry tachycardia, sinus tachycardia, Torsades de Pointes, and ventricular tachycardia.

15 Heart valve disease include aortic valve insufficiency, aortic valve stenosis, hear murmurs, aortic valve prolapse, mitral valve prolapse, tricuspid valve prolapse, mitral valve insufficiency, mitral valve stenosis, pulmonary atresia, pulmonary valve insufficiency, pulmonary valve stenosis, tricuspid atresia, tricuspid valve insufficiency, and tricuspid valve stenosis.

20 Myocardial diseases include alcoholic cardiomyopathy, congestive cardiomyopathy, hypertrophic cardiomyopathy, aortic subvalvular stenosis, pulmonary subvalvular stenosis, restrictive cardiomyopathy, Chagas cardiomyopathy, endocardial fibroelastosis, endomyocardial fibrosis, Kearns Syndrome, myocardial reperfusion injury, and myocarditis.

25 Myocardial ischemias include coronary disease, such as angina pectoris, coronary aneurysm, coronary arteriosclerosis, coronary thrombosis, coronary vasospasm, myocardial infarction and myocardial stunning.

Cardiovascular diseases also include vascular diseases such as aneurysms, angiodyplasia, angiomatosis, bacillary angiomatosis, Hippel-Lindau Disease,  
 30 Klippel-Trenaunay-Weber Syndrome, Sturge-Weber Syndrome, angioneurotic edema, aortic diseases, Takayasu's Arteritis, aortitis, Leriche's Syndrome, arterial occlusive diseases, arteritis, enarteritis, polyarteritis nodosa, cerebrovascular diseases, disorders,

and/or conditions, diabetic angiopathies, diabetic retinopathy, embolisms, thrombosis, erythromelalgia, hemorrhoids, hepatic veno-occlusive disease, hypertension, hypotension, ischemia, peripheral vascular diseases, phlebitis, pulmonary veno-occlusive disease, Raynaud's disease, CREST syndrome, retinal vein occlusion, Scimitar syndrome, superior vena cava syndrome, telangiectasia, atacia telangiectasia, hereditary hemorrhagic telangiectasia, varicocele, varicose veins, varicose ulcer, vasculitis, and venous insufficiency.

Aneurysms include dissecting aneurysms, false aneurysms, infected aneurysms, ruptured aneurysms, aortic aneurysms, cerebral aneurysms, coronary aneurysms, heart aneurysms, and iliac aneurysms.

Arterial occlusive diseases include arteriosclerosis, intermittent claudication, carotid stenosis, fibromuscular dysplasias, mesenteric vascular occlusion, Moyamoya disease, renal artery obstruction, retinal artery occlusion, and thromboangiitis obliterans.

Cerebrovascular diseases, disorders, and/or conditions include carotid artery diseases, cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformation, cerebral artery diseases, cerebral embolism and thrombosis, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, cerebral hemorrhage, epidural hematoma, subdural hematoma, subaraxhnoid hemorrhage, cerebral infarction, cerebral ischemia (including transient), subclavian steal syndrome, periventricular leukomalacia, vascular headache, cluster headache, migraine, and vertebrobasilar insufficiency.

Embolisms include air embolisms, amniotic fluid embolisms, cholesterol embolisms, blue toe syndrome, fat embolisms, pulmonary embolisms, and thromboembolisms. Thrombosis include coronary thrombosis, hepatic vein thrombosis, retinal vein occlusion, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, and thrombophlebitis.

Ischemia includes cerebral ischemia, ischemic colitis, compartment syndromes, anterior compartment syndrome, myocardial ischemia, reperfusion injuries, and peripheral limb ischemia. Vasculitis includes aortitis, arteritis, Behcet's Syndrome, Churg-Strauss Syndrome, mucocutaneous lymph node syndrome,

thromboangiitis obliterans, hypersensitivity vasculitis, Schoenlein-Henoch purpura, allergic cutaneous vasculitis, and Wegener's granulomatosis.

Polynucleotides or polypeptides, or agonists or antagonists of the invention, are especially effective for the treatment of critical limb ischemia and coronary  
5 disease.

Polypeptides may be administered using any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, biolistic injectors, particle accelerators, gelfoam sponge depots, other commercially available depot materials,  
10 osmotic pumps, oral or suppositorial solid pharmaceutical formulations, decanting or topical applications during surgery, aerosol delivery. Such methods are known in the art. Polypeptides of the invention may be administered as part of a Therapeutic, described in more detail below. Methods of delivering polynucleotides of the invention are described in more detail herein.

#### 15 Anti-Angiogenesis Activity

The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate. Rastinejad et al., *Cell* 56:345-355 (1989). In those rare instances in which neovascularization occurs under normal physiological conditions, such as wound  
20 healing, organ regeneration, embryonic development, and female reproductive processes, angiogenesis is stringently regulated and spatially and temporally delimited. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. Unregulated angiogenesis becomes pathologic and sustains progression of many neoplastic and non-neoplastic diseases.  
25 A number of serious diseases are dominated by abnormal neovascularization including solid tumor growth and metastases, arthritis, some types of eye diseases, disorders, and/or conditions, and psoriasis. See, e.g., reviews by Moses et al., *Biotech.* 9:630-634 (1991); Folkman et al., *N. Engl. J. Med.*, 333:1757-1763 (1995); Auerbach et al., *J. Microvasc. Res.* 29:401-411 (1985); Folkman, *Advances in Cancer Research*, eds. Klein and Weinhouse, Academic Press, New York, pp. 175-203 (1985); Patz,  
30 *Am. J. Ophthalmol.* 94:715-743 (1982); and Folkman et al., *Science* 221:719-725 (1983). In a number of pathological conditions, the process of angiogenesis

contributes to the disease state. For example, significant data have accumulated which suggest that the growth of solid tumors is dependent on angiogenesis. Folkman and Klagsbrun, Science 235:442-447 (1987).

5       The present invention provides for treatment of diseases, disorders, and/or conditions associated with neovascularization by administration of the polynucleotides and/or polypeptides of the invention, as well as agonists or antagonists of the present invention. Malignant and metastatic conditions which can be treated with the polynucleotides and polypeptides, or agonists or antagonists of the invention include, but are not limited to, malignancies, solid tumors, and cancers  
10       described herein and otherwise known in the art (for a review of such disorders, see Fishman et al., Medicine, 2d Ed., J. B. Lippincott Co., Philadelphia (1985)). Thus, the present invention provides a method of treating, preventing, and/or diagnosing an angiogenesis-related disease and/or disorder, comprising administering to an individual in need thereof a therapeutically effective amount of a polynucleotide,  
15       polypeptide, antagonist and/or agonist of the invention. For example, polynucleotides, polypeptides, antagonists and/or agonists may be utilized in a variety of additional methods in order to therapeutically treat or prevent a cancer or tumor. Cancers which may be treated, prevented, and/or diagnosed with polynucleotides, polypeptides, antagonists and/or agonists include, but are not limited to solid tumors, including  
20       prostate, lung, breast, ovarian, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, thyroid cancer; primary tumors and metastases; melanomas; glioblastoma; Kaposi's sarcoma; leiomyosarcoma; non- small cell lung cancer; colorectal cancer; advanced malignancies; and blood born tumors such as leukemias. For example,  
25       polynucleotides, polypeptides, antagonists and/or agonists may be delivered topically, in order to treat or prevent cancers such as skin cancer, head and neck tumors, breast tumors, and Kaposi's sarcoma.

      Within yet other aspects, polynucleotides, polypeptides, antagonists and/or agonists may be utilized to treat superficial forms of bladder cancer by, for example,  
30       intravesical administration. Polynucleotides, polypeptides, antagonists and/or agonists may be delivered directly into the tumor, or near the tumor site, via injection or a catheter. Of course, as the artisan of ordinary skill will appreciate, the appropriate



mode of administration will vary according to the cancer to be treated. Other modes of delivery are discussed herein.

Polynucleotides, polypeptides, antagonists and/or agonists may be useful in treating, preventing, and/or diagnosing other diseases, disorders, and/or conditions, besides cancers, which involve angiogenesis. These diseases, disorders, and/or conditions include, but are not limited to: benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; arteriosclerotic plaques; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, uveitis and Pterygia (abnormal blood vessel growth) of the eye; rheumatoid arthritis; psoriasis; delayed wound healing; endometriosis; vasculogenesis; granulations; hypertrophic scars (keloids); nonunion fractures; scleroderma; trachoma; vascular adhesions; myocardial angiogenesis; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; Osler-Webber Syndrome; plaque neovascularization; telangiectasia; hemophilic joints; angiofibroma; fibromuscular dysplasia; wound granulation; Crohn's disease; and atherosclerosis.

For example, within one aspect of the present invention methods are provided for treating, preventing, and/or diagnosing hypertrophic scars and keloids, comprising the step of administering a polynucleotide, polypeptide, antagonist and/or agonist of the invention to a hypertrophic scar or keloid.

Within one embodiment of the present invention polynucleotides, polypeptides, antagonists and/or agonists are directly injected into a hypertrophic scar or keloid, in order to prevent the progression of these lesions. This therapy is of particular value in the prophylactic treatment of conditions which are known to result in the development of hypertrophic scars and keloids (e.g., burns), and is preferably initiated after the proliferative phase has had time to progress (approximately 14 days after the initial injury), but before hypertrophic scar or keloid development. As noted above, the present invention also provides methods for treating, preventing, and/or diagnosing neovascular diseases of the eye, including for example, corneal neovascularization, neovascular glaucoma, proliferative diabetic retinopathy, retrolental fibroplasia and macular degeneration.

Moreover, Ocular diseases, disorders, and/or conditions associated with neovascularization which can be treated, prevented, and/or diagnosed with the polynucleotides and polypeptides of the present invention (including agonists and/or antagonists) include, but are not limited to: neovascular glaucoma, diabetic  
5 retinopathy, retinoblastoma, retrolental fibroplasia, uveitis, retinopathy of prematurity macular degeneration, corneal graft neovascularization, as well as other eye inflammatory diseases, ocular tumors and diseases associated with choroidal or iris neovascularization. See, e.g., reviews by Waltman et al., *Am. J. Ophthalmol.* 85:704-710 (1978) and Gartner et al., *Surv. Ophthalmol.* 22:291-312 (1978).

10 Thus, within one aspect of the present invention methods are provided for treating or preventing neovascular diseases of the eye such as corneal neovascularization (including corneal graft neovascularization), comprising the step of administering to a patient a therapeutically effective amount of a compound (as described above) to the cornea, such that the formation of blood vessels is inhibited.  
15 Briefly, the cornea is a tissue which normally lacks blood vessels. In certain pathological conditions however, capillaries may extend into the cornea from the pericorneal vascular plexus of the limbus. When the cornea becomes vascularized, it also becomes clouded, resulting in a decline in the patient's visual acuity. Visual loss may become complete if the cornea completely opacitates. A wide variety of diseases,  
20 disorders, and/or conditions can result in corneal neovascularization, including for example, corneal infections (e.g., trachoma, herpes simplex keratitis, leishmaniasis and onchocerciasis), immunological processes (e.g., graft rejection and Stevens-Johnson's syndrome), alkali burns, trauma, inflammation (of any cause), toxic and nutritional deficiency states, and as a complication of wearing contact lenses.

25 Within particularly preferred embodiments of the invention, may be prepared for topical administration in saline (combined with any of the preservatives and antimicrobial agents commonly used in ocular preparations), and administered in eyedrop form. The solution or suspension may be prepared in its pure form and administered several times daily. Alternatively, anti-angiogenic compositions,  
30 prepared as described above, may also be administered directly to the cornea. Within preferred embodiments, the anti-angiogenic composition is prepared with a muco-adhesive polymer which binds to cornea. Within further embodiments, the anti-

angiogenic factors or anti-angiogenic compositions may be utilized as an adjunct to conventional steroid therapy. Topical therapy may also be useful prophylactically in corneal lesions which are known to have a high probability of inducing an angiogenic response (such as chemical burns). In these instances the treatment, likely in  
5 combination with steroids, may be instituted immediately to help prevent subsequent complications.

Within other embodiments, the compounds described above may be injected directly into the corneal stroma by an ophthalmologist under microscopic guidance. The preferred site of injection may vary with the morphology of the individual lesion,  
10 but the goal of the administration would be to place the composition at the advancing front of the vasculature (i.e., interspersed between the blood vessels and the normal cornea). In most cases this would involve perilimbic corneal injection to "protect" the cornea from the advancing blood vessels. This method may also be utilized shortly after a corneal insult in order to prophylactically prevent corneal neovascularization.  
15 In this situation the material could be injected in the perilimbic cornea interspersed between the corneal lesion and its undesired potential limbic blood supply. Such methods may also be utilized in a similar fashion to prevent capillary invasion of transplanted corneas. In a sustained-release form injections might only be required 2-3 times per year. A steroid could also be added to the injection solution to reduce  
20 inflammation resulting from the injection itself.

Within another aspect of the present invention, methods are provided for treating or preventing neovascular glaucoma, comprising the step of administering to a patient a therapeutically effective amount of a polynucleotide, polypeptide, antagonist and/or agonist to the eye, such that the formation of blood vessels is  
25 inhibited. In one embodiment, the compound may be administered topically to the eye in order to treat or prevent early forms of neovascular glaucoma. Within other embodiments, the compound may be implanted by injection into the region of the anterior chamber angle. Within other embodiments, the compound may also be placed in any location such that the compound is continuously released into the aqueous  
30 humor. Within another aspect of the present invention, methods are provided for treating or preventing proliferative diabetic retinopathy, comprising the step of administering to a patient a therapeutically effective amount of a polynucleotide,

polypeptide, antagonist and/or agonist to the eyes, such that the formation of blood vessels is inhibited.

Within particularly preferred embodiments of the invention, proliferative diabetic retinopathy may be treated by injection into the aqueous humor or the vitreous, in order to increase the local concentration of the polynucleotide, polypeptide, antagonist and/or agonist in the retina. Preferably, this treatment should be initiated prior to the acquisition of severe disease requiring photocoagulation.

Within another aspect of the present invention, methods are provided for treating or preventing retrolental fibroplasia, comprising the step of administering to a patient a therapeutically effective amount of a polynucleotide, polypeptide, antagonist and/or agonist to the eye, such that the formation of blood vessels is inhibited. The compound may be administered topically, via intravitreal injection and/or via intraocular implants.

Additionally, diseases, disorders, and/or conditions which can be treated, prevented, and/or diagnosed with the polynucleotides, polypeptides, agonists and/or antagonists include, but are not limited to, hemangioma, arthritis, psoriasis, angiofibroma, atherosclerotic plaques, delayed wound healing, granulations, hemophilic joints, hypertrophic scars, nonunion fractures, Osler-Weber syndrome, pyogenic granuloma, scleroderma, trachoma, and vascular adhesions.

Moreover, diseases, disorders, and/or conditions and/or states, which can be treated, prevented, and/or diagnosed with the polynucleotides, polypeptides, agonists and/or antagonists include, but are not limited to, solid tumors, blood born tumors such as leukemias, tumor metastasis, Kaposi's sarcoma, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas, rheumatoid arthritis, psoriasis, ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, and uveitis, delayed wound healing, endometriosis, vasculogenesis, granulations, hypertrophic scars (keloids), nonunion fractures, scleroderma, trachoma, vascular adhesions, myocardial angiogenesis, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, Osler-Webber Syndrome, plaque neovascularization, telangiectasia, hemophilic joints, angiofibroma

fibromuscular dysplasia, wound granulation, Crohn's disease, atherosclerosis, birth control agent by preventing vascularization required for embryo implantation controlling menstruation, diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (Rochele minalia quintosa), ulcers (*Helicobacter pylori*),  
5 Bartonellosis and bacillary angiomatosis.

In one aspect of the birth control method, an amount of the compound sufficient to block embryo implantation is administered before or after intercourse and fertilization have occurred, thus providing an effective method of birth control, possibly a "morning after" method. Polynucleotides, polypeptides, agonists and/or  
10 agonists may also be used in controlling menstruation or administered as either a peritoneal lavage fluid or for peritoneal implantation in the treatment of endometriosis.

Polynucleotides, polypeptides, agonists and/or agonists of the present invention may be incorporated into surgical sutures in order to prevent stitch  
15 granulomas.

Polynucleotides, polypeptides, agonists and/or agonists may be utilized in a wide variety of surgical procedures. For example, within one aspect of the present invention a compositions (in the form of, for example, a spray or film) may be utilized to coat or spray an area prior to removal of a tumor, in order to isolate normal  
20 surrounding tissues from malignant tissue, and/or to prevent the spread of disease to surrounding tissues. Within other aspects of the present invention, compositions (e.g., in the form of a spray) may be delivered via endoscopic procedures in order to coat tumors, or inhibit angiogenesis in a desired locale. Within yet other aspects of the present invention, surgical meshes which have been coated with anti- angiogenic  
25 compositions of the present invention may be utilized in any procedure wherein a surgical mesh might be utilized. For example, within one embodiment of the invention a surgical mesh laden with an anti-angiogenic composition may be utilized during abdominal cancer resection surgery (e.g., subsequent to colon resection) in order to provide support to the structure, and to release an amount of the anti-  
30 angiogenic factor.

Within further aspects of the present invention, methods are provided for treating tumor excision sites, comprising administering a polynucleotide, polypeptide,

agonist and/or agonist to the resection margins of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new blood vessels at the site is inhibited. Within one embodiment of the invention, the anti-angiogenic compound is administered directly to the tumor excision site (e.g., applied by swabbing, brushing or otherwise coating the resection margins of the tumor with the anti-angiogenic compound). Alternatively, the anti-angiogenic compounds may be incorporated into known surgical pastes prior to administration. Within particularly preferred embodiments of the invention, the anti-angiogenic compounds are applied after hepatic resections for malignancy, and after neurosurgical operations.

10        Within one aspect of the present invention, polynucleotides, polypeptides, agonists and/or agonists may be administered to the resection margin of a wide variety of tumors, including for example, breast, colon, brain and hepatic tumors. For example, within one embodiment of the invention, anti-angiogenic compounds may be administered to the site of a neurological tumor subsequent to excision, such that  
15        the formation of new blood vessels at the site are inhibited.

      The polynucleotides, polypeptides, agonists and/or agonists of the present invention may also be administered along with other anti-angiogenic factors. Representative examples of other anti-angiogenic factors include: Anti-Invasive Factor, retinoic acid and derivatives thereof, paclitaxel, Suramin, Tissue Inhibitor of  
20        Metalloproteinase-1, Tissue Inhibitor of Metalloproteinase-2, Plasminogen Activator Inhibitor-1, Plasminogen Activator Inhibitor-2, and various forms of the lighter "d group" transition metals.

      Lighter "d group" transition metals include, for example, vanadium, molybdenum, tungsten, titanium, niobium, and tantalum species. Such transition  
25        metal species may form transition metal complexes. Suitable complexes of the above-mentioned transition metal species include oxo transition metal complexes.

      Representative examples of vanadium complexes include oxo vanadium complexes such as vanadate and vanadyl complexes. Suitable vanadate complexes include metavanadate and orthovanadate complexes such as, for example, ammonium  
30        metavanadate, sodium metavanadate, and sodium orthovanadate. Suitable vanadyl complexes include, for example, vanadyl acetylacetonate and vanadyl sulfate including vanadyl sulfate hydrates such as vanadyl sulfate mono- and trihydrates.

Representative examples of tungsten and molybdenum complexes also include oxo complexes. Suitable oxo tungsten complexes include tungstate and tungsten oxide complexes. Suitable tungstate complexes include ammonium tungstate, calcium tungstate, sodium tungstate dihydrate, and tungstic acid. Suitable tungsten oxides include tungsten (IV) oxide and tungsten (VI) oxide. Suitable oxo molybdenum complexes include molybdate, molybdenum oxide, and molybdenyl complexes. Suitable molybdate complexes include ammonium molybdate and its hydrates, sodium molybdate and its hydrates, and potassium molybdate and its hydrates. Suitable molybdenum oxides include molybdenum (VI) oxide, molybdenum (VI) oxide, and molybdic acid. Suitable molybdenyl complexes include, for example, molybdenyl acetylacetonate. Other suitable tungsten and molybdenum complexes include hydroxo derivatives derived from, for example, glycerol, tartaric acid, and sugars.

A wide variety of other anti-angiogenic factors may also be utilized within the context of the present invention. Representative examples include platelet factor 4; protamine sulphate; sulphated chitin derivatives (prepared from queen crab shells), (Murata et al., Cancer Res. 51:22-26, 1991); Sulphated Polysaccharide Peptidoglycan Complex (SP- PG) (the function of this compound may be enhanced by the presence of steroids such as estrogen, and tamoxifen citrate); Staurosporine; modulators of matrix metabolism, including for example, proline analogs, cishydroxyproline, d,L-3,4-dehydroproline, Thiaproline, alpha,alpha-dipyridyl, aminopropionitrile fumarate; 4-propyl-5-(4-pyridinyl)-2(3H)-oxazolone; Methotrexate; Mitoxantrone; Heparin; Interferons; 2 Macroglobulin-serum; ChIMP-3 (Pavloff et al., J. Bio. Chem. 267:17321-17326, 1992); Chymostatin (Tomkinson et al., Biochem J. 286:475-480, 1992); Cyclodextrin Tetradecasulfate; Eponemycin; Camptothecin; Fumagillin (Ingber et al., Nature 348:555-557, 1990); Gold Sodium Thiomalate ("GST"; Matsubara and Ziff, J. Clin. Invest. 79:1440-1446, 1987); anticollagenase-serum; alpha2-antiplasmin (Holmes et al., J. Biol. Chem. 262(4):1659-1664, 1987); Bisantrone (National Cancer Institute); Lobenzarit disodium (N-(2)-carboxyphenyl-4-chloroanthronilic acid disodium or "CCA"; Takeuchi et al., Agents Actions 36:312-316, 1992); Thalidomide; Angostatic steroid; AGM-1470; carboxynaminolmidazole; and metalloproteinase inhibitors such as BB94.

## Diseases at the Cellular Level

Diseases associated with increased cell survival or the inhibition of apoptosis that could be treated, prevented, and/or diagnosed by the polynucleotides or polypeptides and/or antagonists or agonists of the invention, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, including, but not limited to colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma, osteoclastoma, osteosarcoma, chondrosarcoma, adenoma, breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune diseases, disorders, and/or conditions (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection. In preferred embodiments, the polynucleotides or polypeptides, and/or agonists or antagonists of the invention are used to inhibit growth, progression, and/or metastasis of cancers, in particular those listed above.

Additional diseases or conditions associated with increased cell survival that could be treated, prevented or diagnosed by the polynucleotides or polypeptides, or agonists or antagonists of the invention, include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic



cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, and retinoblastoma.

10 Diseases associated with increased apoptosis that could be treated, prevented, and/or diagnosed by the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, include AIDS; neurodegenerative diseases, disorders, and/or conditions (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration and brain tumor or prior associated disease); autoimmune diseases, disorders, and/or conditions (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) myelodysplastic syndromes (such as aplastic anemia), graft v. host disease, ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), liver injury (e.g., hepatitis related liver injury, ischemia/reperfusion injury, cholestasis (bile duct injury) and liver cancer); toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

#### Wound Healing and Epithelial Cell Proliferation

25 In accordance with yet a further aspect of the present invention, there is provided a process for utilizing the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, for therapeutic purposes, for example, to stimulate epithelial cell proliferation and basal keratinocytes for the purpose of wound healing, and to stimulate hair follicle production and healing of dermal wounds.

30 Polynucleotides or polypeptides, as well as agonists or antagonists of the invention, may be clinically useful in stimulating wound healing including surgical wounds, excisional wounds, deep wounds involving damage of the dermis and epidermis, eye

tissue wounds, dental tissue wounds, oral cavity wounds, diabetic ulcers, dermal ulcers, cubitus ulcers, arterial ulcers, venous stasis ulcers, burns resulting from heat exposure or chemicals, and other abnormal wound healing conditions such as uremia, malnutrition, vitamin deficiencies and complications associated with systemic treatment with steroids, radiation therapy and antineoplastic drugs and antimetabolites. Polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used to promote dermal reestablishment subsequent to dermal loss.

The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used to increase the adherence of skin grafts to a wound bed and to stimulate re-epithelialization from the wound bed. The following are a non-exhaustive list of grafts that polynucleotides or polypeptides, agonists or antagonists of the invention, could be used to increase adherence to a wound bed: autografts, artificial skin, allografts, autodermic graft, autoepidermic grafts, avacular grafts, Blair-Brown grafts, bone graft, brephoplastic grafts, cutis graft, delayed graft, dermic graft, epidermic graft, fascia graft, full thickness graft, heterologous graft, xenograft, homologous graft, hyperplastic graft, lamellar graft, mesh graft, mucosal graft, Ollier-Thiersch graft, omenpal graft, patch graft, pedicle graft, penetrating graft, split skin graft, thick split graft. The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, can be used to promote skin strength and to improve the appearance of aged skin.

It is believed that the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, will also produce changes in hepatocyte proliferation, and epithelial cell proliferation in the lung, breast, pancreas, stomach, small intestine, and large intestine. The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could promote proliferation of epithelial cells such as sebocytes, hair follicles, hepatocytes, type II pneumocytes, mucin-producing goblet cells, and other epithelial cells and their progenitors contained within the skin, lung, liver, and gastrointestinal tract. The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, may promote proliferation of endothelial cells, keratinocytes, and basal keratinocytes.

The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could also be used to reduce the side effects of gut toxicity that result from radiation, chemotherapy treatments or viral infections. The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, may have a cytoprotective effect on the small intestine mucosa. The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, may also stimulate healing of mucositis (mouth ulcers) that result from chemotherapy and viral infections.

The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could further be used in full regeneration of skin in full and partial thickness skin defects, including burns, (i.e., repopulation of hair follicles, sweat glands, and sebaceous glands), treatment of other skin defects such as psoriasis. The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used to treat epidermolysis bullosa, a defect in adherence of the epidermis to the underlying dermis which results in frequent, open and painful blisters by accelerating reepithelialization of these lesions. The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could also be used to treat gastric and duodenal ulcers and help heal by scar formation of the mucosal lining and regeneration of glandular mucosa and duodenal mucosal lining more rapidly. Inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, are diseases which result in destruction of the mucosal surface of the small or large intestine, respectively. Thus, the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used to promote the resurfacing of the mucosal surface to aid more rapid healing and to prevent progression of inflammatory bowel disease. Treatment with the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, is expected to have a significant effect on the production of mucus throughout the gastrointestinal tract and could be used to protect the intestinal mucosa from injurious substances that are ingested or following surgery. The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used to treat diseases associated with the under expression of the polynucleotides of the invention.

Moreover, the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used to prevent and heal damage to the lungs due to various pathological states. A tubulin tyrosine ligase protein such as the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, which could stimulate proliferation and differentiation and promote the repair of alveoli and bronchiolar epithelium to prevent or treat acute or chronic lung damage. For example, emphysema, which results in the progressive loss of aveoli, and inhalation injuries, i.e., resulting from smoke inhalation and burns, that cause necrosis of the bronchiolar epithelium and alveoli could be effectively treated, prevented, and/or diagnosed using the polynucleotides or polypeptides, and/or agonists or antagonists of the invention. Also, the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used to stimulate the proliferation of and differentiation of type II pneumocytes, which may help treat or prevent disease such as hyaline membrane diseases, such as infant respiratory distress syndrome and bronchopulmonary displasia, in premature infants.

The polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could stimulate the proliferation and differentiation of hepatocytes and, thus, could be used to alleviate or treat liver diseases and pathologies such as fulminant liver failure caused by cirrhosis, liver damage caused by viral hepatitis and toxic substances (i.e., acetaminophen, carbon tetraholoride and other hepatotoxins known in the art).

In addition, the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used treat or prevent the onset of diabetes mellitus. In patients with newly diagnosed Types I and II diabetes, where some islet cell function remains, the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used to maintain the islet function so as to alleviate, delay or prevent permanent manifestation of the disease. Also, the polynucleotides or polypeptides, and/or agonists or antagonists of the invention, could be used as an auxiliary in islet cell transplantation to improve or promote islet cell function.

#### Infectious Disease

A polypeptide or polynucleotide and/or agonist or antagonist of the present invention can be used to treat, prevent, and/or diagnose infectious agents. For

example, by increasing the immune response, particularly increasing the proliferation and differentiation of B and/or T cells, infectious diseases may be treated, prevented, and/or diagnosed. The immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively,  
 5 polypeptide or polynucleotide and/or agonist or antagonist of the present invention may also directly inhibit the infectious agent, without necessarily eliciting an immune response.

Viruses are one example of an infectious agent that can cause disease or symptoms that can be treated, prevented, and/or diagnosed by a polynucleotide or  
 10 polypeptide and/or agonist or antagonist of the present invention. Examples of viruses, include, but are not limited to Examples of viruses, include, but are not limited to the following DNA and RNA viruses and viral families: Arbovirus, Adenoviridae, Arenaviridae, Arterivirus, Birnaviridae, Bunyaviridae, Caliciviridae, Circoviridae, Coronaviridae, Dengue, EBV, HIV, Flaviviridae, Hepadnaviridae  
 15 (Hepatitis), Herpesviridae (such as, Cytomegalovirus, Herpes Simplex, Herpes Zoster), Mononegavirus (e.g., Paramyxoviridae, Morbillivirus, Rhabdoviridae), Orthomyxoviridae (e.g., Influenza A, Influenza B, and parainfluenza), Papiloma virus, Papovaviridae, Parvoviridae, Picornaviridae, Poxviridae (such as Smallpox or Vaccinia), Reoviridae (e.g., Rotavirus), Retroviridae (HTLV-I, HTLV-II, Lentivirus),  
 20 and Togaviridae (e.g., Rubivirus). Viruses falling within these families can cause a variety of diseases or symptoms, including, but not limited to: arthritis, bronchiolitis, respiratory syncytial virus, encephalitis, eye infections (e.g., conjunctivitis, keratitis), chronic fatigue syndrome, hepatitis (A, B, C, E, Chronic Active, Delta), Japanese B encephalitis, Junin, Chikungunya, Rift Valley fever, yellow fever, meningitis,  
 25 opportunistic infections (e.g., AIDS), pneumonia, Burkitt's Lymphoma, chickenpox, hemorrhagic fever, Measles, Mumps, Parainfluenza, Rabies, the common cold, Polio, leukemia, Rubella, sexually transmitted diseases, skin diseases (e.g., Kaposi's, warts), and viremia. polynucleotides or polypeptides, or agonists or antagonists of the invention, can be used to treat, prevent, and/or diagnose any of these symptoms or  
 30 diseases. In specific embodiments, polynucleotides, polypeptides, or agonists or antagonists of the invention are used to treat, prevent, and/or diagnose: meningitis, Dengue, EBV, and/or hepatitis (e.g., hepatitis B). In an additional specific

embodiment polynucleotides, polypeptides, or agonists or antagonists of the invention are used to treat patients nonresponsive to one or more other commercially available hepatitis vaccines. In a further specific embodiment polynucleotides, polypeptides, or agonists or antagonists of the invention are used to treat, prevent, and/or diagnose  
 5 AIDS.

Similarly, bacterial or fungal agents that can cause disease or symptoms and that can be treated, prevented, and/or diagnosed by a polynucleotide or polypeptide and/or agonist or antagonist of the present invention include, but not limited to, include, but not limited to, the following Gram-Negative and Gram-positive bacteria  
 10 and bacterial families and fungi: Actinomycetales (e.g., Corynebacterium, Mycobacterium, Norcardia), Cryptococcus neoformans, Aspergillosis, Bacillaceae (e.g., Anthrax, Clostridium), Bacteroidaceae, Blastomycosis, Bordetella, Borrelia (e.g., Borrelia burgdorferi), Brucellosis, Candidiasis, Campylobacter, Coccidioidomycosis, Cryptococcosis, Dermatocycoses, E. coli (e.g., Enterotoxigenic  
 15 E. coli and Enterohemorrhagic E. coli), Enterobacteriaceae (Klebsiella, Salmonella (e.g., Salmonella typhi, and Salmonella paratyphi), Serratia, Yersinia), Erysipelothrix, Helicobacter, Legionellosis, Leptospirosis, Listeria, Mycoplasmatales, Mycobacterium leprae, Vibrio cholerae, Neisseriaceae (e.g., Acinetobacter, Gonorrhea, Meningococcal), Meisseria meningitidis, Pasteurellacea Infections (e.g.,  
 20 Actinobacillus, Heamophilus (e.g., Heamophilus influenza type B), Pasteurella), Pseudomonas, Rickettsiaceae, Chlamydiaceae, Syphilis, Shigella spp., Staphylococcal, Meningiococcal, Pneumococcal and Streptococcal (e.g., Streptococcus pneumoniae and Group B Streptococcus). These bacterial or fungal families can cause the following diseases or symptoms, including, but not limited to:  
 25 bacteremia, endocarditis, eye infections (conjunctivitis, tuberculosis, uveitis), gingivitis, opportunistic infections (e.g., AIDS related infections), paronychia, prosthesis-related infections, Reiter's Disease, respiratory tract infections, such as Whooping Cough or Empyema, sepsis, Lyme Disease, Cat-Scratch Disease, Dysentery, Paratyphoid Fever, food poisoning, Typhoid, pneumonia, Gonorrhea, meningitis (e.g., meningitis types A and B), Chlamydia, Syphilis, Diphtheria, Leprosy,  
 30 Paratuberculosis, Tuberculosis, Lupus, Botulism, gangrene, tetanus, impetigo, Rheumatic Fever, Scarlet Fever, sexually transmitted diseases, skin diseases (e.g.,

cellulitis, dermatocycoses), toxemia, urinary tract infections, wound infections. Polynucleotides or polypeptides, agonists or antagonists of the invention, can be used to treat, prevent, and/or diagnose any of these symptoms or diseases. In specific embodiments, polynucleotides, polypeptides, agonists or antagonists of the invention  
 5 are used to treat, prevent, and/or diagnose: tetanus, Diphtheria, botulism, and/or meningitis type B.

Moreover, parasitic agents causing disease or symptoms that can be treated, prevented, and/or diagnosed by a polynucleotide or polypeptide and/or agonist or antagonist of the present invention include, but not limited to, the following families  
 10 or class: Amebiasis, Babesiosis, Coccidiosis, Cryptosporidiosis, Dientamoebiasis, Dourine, Ectoparasitic, Giardiasis, Helminthiasis, Leishmaniasis, Theileriasis, Toxoplasmosis, Trypanosomiasis, and Trichomonas and Sporozoans (e.g., Plasmodium virax, Plasmodium falciparum, Plasmodium malariae and Plasmodium ovale). These parasites can cause a variety of diseases or symptoms, including, but not  
 15 limited to: Scabies, Trombiculiasis, eye infections, intestinal disease (e.g., dysentery, giardiasis), liver disease, lung disease, opportunistic infections (e.g., AIDS related), malaria, pregnancy complications, and toxoplasmosis. polynucleotides or polypeptides, or agonists or antagonists of the invention, can be used to treat, prevent, and/or diagnose any of these symptoms or diseases. In specific embodiments,  
 20 polynucleotides, polypeptides, or agonists or antagonists of the invention are used to treat, prevent, and/or diagnose malaria.

Preferably, treatment or prevention using a polypeptide or polynucleotide and/or agonist or antagonist of the present invention could either be by administering an effective amount of a polypeptide to the patient, or by removing cells from the  
 25 patient, supplying the cells with a polynucleotide of the present invention, and returning the engineered cells to the patient (ex vivo therapy). Moreover, the polypeptide or polynucleotide of the present invention can be used as an antigen in a vaccine to raise an immune response against infectious disease.

#### Regeneration

30 A polynucleotide or polypeptide and/or agonist or antagonist of the present invention can be used to differentiate, proliferate, and attract cells, leading to the regeneration of tissues. (See, Science 276:59-87 (1997).) The regeneration of tissues

could be used to repair, replace, or protect tissue damaged by congenital defects, trauma (wounds, burns, incisions, or ulcers), age, disease (e.g. osteoporosis, osteoarthritis, periodontal disease, liver failure), surgery, including cosmetic plastic surgery, fibrosis, reperfusion injury, or systemic cytokine damage.

5           Tissues that could be regenerated using the present invention include organs (e.g., pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac), vasculature (including vascular and lymphatics), nervous, hematopoietic, and skeletal (bone, cartilage, tendon, and ligament) tissue. Preferably, regeneration occurs without or decreased scarring. Regeneration also may include angiogenesis.

10           Moreover, a polynucleotide or polypeptide and/or agonist or antagonist of the present invention may increase regeneration of tissues difficult to heal. For example, increased tendon/ligament regeneration would quicken recovery time after damage. A polynucleotide or polypeptide and/or agonist or antagonist of the present invention could also be used prophylactically in an effort to avoid damage. Specific diseases  
15           that could be treated, prevented, and/or diagnosed include of tendinitis, carpal tunnel syndrome, and other tendon or ligament defects. A further example of tissue regeneration of non-healing wounds includes pressure ulcers, ulcers associated with vascular insufficiency, surgical, and traumatic wounds.

            Similarly, nerve and brain tissue could also be regenerated by using a  
20           polynucleotide or polypeptide and/or agonist or antagonist of the present invention to proliferate and differentiate nerve cells. Diseases that could be treated, prevented, and/or diagnosed using this method include central and peripheral nervous system diseases, neuropathies, or mechanical and traumatic diseases, disorders, and/or conditions (e.g., spinal cord disorders, head trauma, cerebrovascular disease, and  
25           stroke). Specifically, diseases associated with peripheral nerve injuries, peripheral neuropathy (e.g., resulting from chemotherapy or other medical therapies), localized neuropathies, and central nervous system diseases (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome), could all be treated, prevented, and/or diagnosed using the  
30           polynucleotide or polypeptide and/or agonist or antagonist of the present invention.



## Binding Activity

A polypeptide of the present invention may be used to screen for molecules that bind to the polypeptide or for molecules to which the polypeptide binds. The binding of the polypeptide and the molecule may activate (agonist), increase, inhibit  
5 (antagonist), or decrease activity of the polypeptide or the molecule bound. Examples of such molecules include antibodies, oligonucleotides, proteins (e.g., receptors), or small molecules.

Preferably, the molecule is closely related to the natural ligand of the polypeptide, e.g., a fragment of the ligand, or a natural substrate, a ligand, a structural  
10 or functional mimetic. (See, Coligan et al., Current Protocols in Immunology 1(2):Chapter 5 (1991).) Similarly, the molecule can be closely related to the natural receptor to which the polypeptide binds, or at least, a fragment of the receptor capable of being bound by the polypeptide (e.g., active site). In either case, the molecule can be rationally designed using known techniques.

15 Preferably, the screening for these molecules involves producing appropriate cells which express the polypeptide, either as a secreted protein or on the cell membrane. Preferred cells include cells from mammals, yeast, Drosophila, or E. coli. Cells expressing the polypeptide (or cell membrane containing the expressed polypeptide) are then preferably contacted with a test compound potentially  
20 containing the molecule to observe binding, stimulation, or inhibition of activity of either the polypeptide or the molecule.

The assay may simply test binding of a candidate compound to the polypeptide, wherein binding is detected by a label, or in an assay involving competition with a labeled competitor. Further, the assay may test whether the  
25 candidate compound results in a signal generated by binding to the polypeptide.

Alternatively, the assay can be carried out using cell-free preparations, polypeptide/molecule affixed to a solid support, chemical libraries, or natural product mixtures. The assay may also simply comprise the steps of mixing a candidate compound with a solution containing a polypeptide, measuring polypeptide/molecule  
30 activity or binding, and comparing the polypeptide/molecule activity or binding to a standard.

Preferably, an ELISA assay can measure polypeptide level or activity in a sample (e.g., biological sample) using a monoclonal or polyclonal antibody. The antibody can measure polypeptide level or activity by either binding, directly or indirectly, to the polypeptide or by competing with the polypeptide for a substrate.

5        Additionally, the receptor to which a polypeptide of the invention binds can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). For example, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to the polypeptides, for  
10        example, NIH3T3 cells which are known to contain multiple receptors for the FGF family proteins, and SC-3 cells, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the polypeptides. Transfected cells which are grown on glass slides are exposed to the polypeptide of the present invention, after they have been labeled. The polypeptides  
15        can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase.

Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an iterative sub-pooling and re-screening process, eventually  
20        yielding a single clones that encodes the putative receptor.

As an alternative approach for receptor identification, the labeled polypeptides can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE analysis and exposed to X-ray film. The labeled complex containing the receptors of the  
25        polypeptides can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the genes encoding the putative receptors.

Moreover, the techniques of gene-shuffling, motif-shuffling, exon-shuffling,  
30        and/or codon-shuffling (collectively referred to as "DNA shuffling") may be employed to modulate the activities of polypeptides of the invention thereby effectively generating agonists and antagonists of polypeptides of the invention. See

generally, U.S. Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, and 5,837,458, and Patten, P. A., et al., *Curr. Opinion Biotechnol.* 8:724-33 (1997); Harayama, S. *Trends Biotechnol.* 16(2):76-82 (1998); Hansson, L. O., et al., *J. Mol. Biol.* 287:265-76 (1999); and Lorenzo, M. M. and Blasco, R. *Biotechniques* 5 24(2):308-13 (1998) (each of these patents and publications are hereby incorporated by reference). In one embodiment, alteration of polynucleotides and corresponding polypeptides of the invention may be achieved by DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments into a desired polynucleotide sequence of the invention molecule by homologous, or site-specific, recombination.

10 In another embodiment, polynucleotides and corresponding polypeptides of the invention may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of the polypeptides of the invention may be recombined with one or more

15 components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules. In preferred embodiments, the heterologous molecules are family members. In further preferred embodiments, the heterologous molecule is a tubulin tyrosine ligase protein such as, for example, platelet-derived tubulin tyrosine ligase protein (PDGF), insulin-like tubulin tyrosine ligase protein (IGF-I),

20 transforming tubulin tyrosine ligase protein (TGF)-alpha, epidermal tubulin tyrosine ligase protein (EGF), fibroblast tubulin tyrosine ligase protein (FGF), TGF-beta, bone morphogenetic protein (BMP)-2, BMP-4, BMP-5, BMP-6, BMP-7, activins A and B, decapentaplegic(dpp), 60A, OP-2, dorsalin, growth differentiation factors (GDFs), nodal, MIS, inhibin-alpha, TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta5, and glial-

25 derived neurotrophic factor (GDNF).

Other preferred fragments are biologically active fragments of the polypeptides of the invention. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of the polypeptide. The biological activity of the fragments may include an improved desired activity, or a

30 decreased undesirable activity.

Additionally, this invention provides a method of screening compounds to identify those which modulate the action of the polypeptide of the present invention.

An example of such an assay comprises combining a mammalian fibroblast cell, a the polypeptide of the present invention, the compound to be screened and 3[H] thymidine under cell culture conditions where the fibroblast cell would normally proliferate. A control assay may be performed in the absence of the compound to be  
5 screened and compared to the amount of fibroblast proliferation in the presence of the compound to determine if the compound stimulates proliferation by determining the uptake of 3[H] thymidine in each case. The amount of fibroblast cell proliferation is measured by liquid scintillation chromatography which measures the incorporation of 3[H] thymidine. Both agonist and antagonist compounds may be identified by this  
10 procedure.

In another method, a mammalian cell or membrane preparation expressing a receptor for a polypeptide of the present invention is incubated with a labeled polypeptide of the present invention in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured.  
15 Alternatively, the response of a known second messenger system following interaction of a compound to be screened and the receptor is measured and the ability of the compound to bind to the receptor and elicit a second messenger response is measured to determine if the compound is a potential agonist or antagonist. Such second messenger systems include but are not limited to, cAMP guanylate cyclase,  
20 ion channels or phosphoinositide hydrolysis.

All of these above assays can be used as diagnostic or prognostic markers. The molecules discovered using these assays can be used to treat, prevent, and/or diagnose disease or to bring about a particular result in a patient (e.g., blood vessel growth) by activating or inhibiting the polypeptide/molecule. Moreover, the assays can discover  
25 agents which may inhibit or enhance the production of the polypeptides of the invention from suitably manipulated cells or tissues. Therefore, the invention includes a method of identifying compounds which bind to the polypeptides of the invention comprising the steps of: (a) incubating a candidate binding compound with the polypeptide; and (b) determining if binding has occurred. Moreover, the invention  
30 includes a method of identifying agonists/antagonists comprising the steps of: (a) incubating a candidate compound with the polypeptide, (b) assaying a biological

activity, and (c) determining if a biological activity of the polypeptide has been altered.

Also, one could identify molecules bind a polypeptide of the invention experimentally by using the beta-pleated sheet regions contained in the polypeptide sequence of the protein. Accordingly, specific embodiments of the invention are directed to polynucleotides encoding polypeptides which comprise, or alternatively consist of, the amino acid sequence of each beta pleated sheet regions in a disclosed polypeptide sequence. Additional embodiments of the invention are directed to polynucleotides encoding polypeptides which comprise, or alternatively consist of, any combination or all of contained in the polypeptide sequences of the invention. Additional preferred embodiments of the invention are directed to polypeptides which comprise, or alternatively consist of, the amino acid sequence of each of the beta pleated sheet regions in one of the polypeptide sequences of the invention. Additional embodiments of the invention are directed to polypeptides which comprise, or alternatively consist of, any combination or all of the beta pleated sheet regions in one of the polypeptide sequences of the invention.

#### Targeted Delivery

In another embodiment, the invention provides a method of delivering compositions to targeted cells expressing a receptor for a polypeptide of the invention, or cells expressing a cell bound form of a polypeptide of the invention.

As discussed herein, polypeptides or antibodies of the invention may be associated with heterologous polypeptides, heterologous nucleic acids, toxins, or prodrugs via hydrophobic, hydrophilic, ionic and/or covalent interactions. In one embodiment, the invention provides a method for the specific delivery of compositions of the invention to cells by administering polypeptides of the invention (including antibodies) that are associated with heterologous polypeptides or nucleic acids. In one example, the invention provides a method for delivering a therapeutic protein into the targeted cell. In another example, the invention provides a method for delivering a single stranded nucleic acid (e.g., antisense or ribozymes) or double stranded nucleic acid (e.g., DNA that can integrate into the cell's genome or replicate episomally and that can be transcribed) into the targeted cell.

In another embodiment, the invention provides a method for the specific destruction of cells (e.g., the destruction of tumor cells) by administering polypeptides of the invention (e.g., polypeptides of the invention or antibodies of the invention) in association with toxins or cytotoxic prodrugs.

5 By "toxin" is meant compounds that bind and activate endogenous cytotoxic effector systems, radioisotopes, holotoxins, modified toxins, catalytic subunits of toxins, or any molecules or enzymes not normally present in or on the surface of a cell that under defined conditions cause the cell's death. Toxins that may be used according to the methods of the invention include, but are not limited to, radioisotopes  
10 known in the art, compounds such as, for example, antibodies (or complement fixing containing portions thereof) that bind an inherent or induced endogenous cytotoxic effector system, thymidine kinase, endonuclease, RNase, alpha toxin, ricin, abrin, Pseudomonas exotoxin A, diphtheria toxin, saporin, momordin, gelonin, pokeweed antiviral protein, alpha-sarcin and cholera toxin. By "cytotoxic prodrug" is meant a  
15 non-toxic compound that is converted by an enzyme, normally present in the cell, into a cytotoxic compound. Cytotoxic prodrugs that may be used according to the methods of the invention include, but are not limited to, glutamyl derivatives of benzoic acid mustard alkylating agent, phosphate derivatives of etoposide or mitomycin C, cytosine arabinoside, daunorubisin, and phenoxyacetamide derivatives of  
20 doxorubicin.

#### Drug Screening

Further contemplated is the use of the polypeptides of the present invention, or the polynucleotides encoding these polypeptides, to screen for molecules which modify the activities of the polypeptides of the present invention. Such a method  
25 would include contacting the polypeptide of the present invention with a selected compound(s) suspected of having antagonist or agonist activity, and assaying the activity of these polypeptides following binding.

This invention is particularly useful for screening therapeutic compounds by using the polypeptides of the present invention, or binding fragments thereof, in any  
30 of a variety of drug screening techniques. The polypeptide or fragment employed in such a test may be affixed to a solid support, expressed on a cell surface, free in solution, or located intracellularly. One method of drug screening utilizes eukaryotic

or prokaryotic host cells which are stably transformed with recombinant nucleic acids expressing the polypeptide or fragment. Drugs are screened against such transformed cells in competitive binding assays. One may measure, for example, the formulation of complexes between the agent being tested and a polypeptide of the present invention.

Thus, the present invention provides methods of screening for drugs or any other agents which affect activities mediated by the polypeptides of the present invention. These methods comprise contacting such an agent with a polypeptide of the present invention or a fragment thereof and assaying for the presence of a complex between the agent and the polypeptide or a fragment thereof, by methods well known in the art. In such a competitive binding assay, the agents to screen are typically labeled. Following incubation, free agent is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of a particular agent to bind to the polypeptides of the present invention.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to the polypeptides of the present invention, and is described in great detail in European Patent Application 84/03564, published on September 13, 1984, which is incorporated herein by reference herein. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with polypeptides of the present invention and washed. Bound polypeptides are then detected by methods well known in the art. Purified polypeptides are coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies may be used to capture the peptide and immobilize it on the solid support.

This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding polypeptides of the present invention specifically compete with a test compound for binding to the polypeptides or fragments thereof. In this manner, the antibodies are used to detect the presence of any peptide which shares one or more antigenic epitopes with a polypeptide of the invention.

The human BGS-42 polypeptides and/or peptides of the present invention, or immunogenic fragments or oligopeptides thereof, can be used for screening therapeutic drugs or compounds in a variety of drug screening techniques. The fragment employed in such a screening assay may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The reduction or abolition of activity of the formation of binding complexes between the ion channel protein and the agent being tested can be measured. Thus, the present invention provides a method for screening or assessing a plurality of compounds for their specific binding affinity with a BGS-42 polypeptide, or a bindable peptide fragment, of this invention, comprising providing a plurality of compounds, combining the BGS-42 polypeptide, or a bindable peptide fragment, with each of a plurality of compounds for a time sufficient to allow binding under suitable conditions and detecting binding of the BGS-42 polypeptide or peptide to each of the plurality of test compounds, thereby identifying the compounds that specifically bind to the BGS-42 polypeptide or peptide.

Methods of identifying compounds that modulate the activity of the novel human BGS-42 polypeptides and/or peptides are provided by the present invention and comprise combining a potential or candidate compound or drug modulator of tubulin tyrosine ligase protein biological activity with an BGS-42 polypeptide or peptide, for example, the BGS-42 amino acid sequence as set forth in SEQ ID NOS:2, and measuring an effect of the candidate compound or drug modulator on the biological activity of the BGS-42 polypeptide or peptide. Such measurable effects include, for example, physical binding interaction; the ability to cleave a suitable tubulin tyrosine ligase protein substrate; effects on native and cloned BGS-42-expressing cell line; and effects of modulators or other tubulin tyrosine ligase protein-mediated physiological measures.

Another method of identifying compounds that modulate the biological activity of the novel BGS-42 polypeptides of the present invention comprises combining a potential or candidate compound or drug modulator of a tubulin tyrosine ligase protein biological activity with a host cell that expresses the BGS-42 polypeptide and measuring an effect of the candidate compound or drug modulator on the biological activity of the BGS-42 polypeptide. The host cell can also be capable of



being induced to express the BGS-42 polypeptide, e.g., via inducible expression. Physiological effects of a given modulator candidate on the BGS-42 polypeptide can also be measured. Thus, cellular assays for particular tubulin tyrosine ligase protein modulators may be either direct measurement or quantification of the physical  
5 biological activity of the BGS-42 polypeptide, or they may be measurement or quantification of a physiological effect. Such methods preferably employ a BGS-42 polypeptide as described herein, or an overexpressed recombinant BGS-42 polypeptide in suitable host cells containing an expression vector as described herein, wherein the BGS-42 polypeptide is expressed, overexpressed, or undergoes  
10 upregulated expression.

Another aspect of the present invention embraces a method of screening for a compound that is capable of modulating the biological activity of a BGS-42 polypeptide, comprising providing a host cell containing an expression vector harboring a nucleic acid sequence encoding a BGS-42 polypeptide, or a functional  
15 peptide or portion thereof (e.g., SEQ ID NOS:2); determining the biological activity of the expressed BGS-42 polypeptide in the absence of a modulator compound; contacting the cell with the modulator compound and determining the biological activity of the expressed BGS-42 polypeptide in the presence of the modulator compound. In such a method, a difference between the activity of the BGS-42  
20 polypeptide in the presence of the modulator compound and in the absence of the modulator compound indicates a modulating effect of the compound.

Essentially any chemical compound can be employed as a potential modulator or ligand in the assays according to the present invention. Compounds tested as tubulin tyrosine ligase protein modulators can be any small chemical compound, or  
25 biological entity (e.g., protein, sugar, nucleic acid, lipid). Test compounds will typically be small chemical molecules and peptides. Generally, the compounds used as potential modulators can be dissolved in aqueous or organic (e.g., DMSO-based) solutions. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source. Assays are  
30 typically run in parallel, for example, in microtiter formats on microtiter plates in robotic assays. There are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka

Chemika-Biochemica Analytika (Buchs, Switzerland), for example. Also, compounds may be synthesized by methods known in the art.

High throughput screening methodologies are particularly envisioned for the detection of modulators of the novel BGS-42 polynucleotides and polypeptides described herein. Such high throughput screening methods typically involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (e.g., ligand or modulator compounds). Such combinatorial chemical libraries or ligand libraries are then screened in one or more assays to identify those library members (e.g., particular chemical species or subclasses) that display a desired characteristic activity. The compounds so identified can serve as conventional lead compounds, or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated either by chemical synthesis or biological synthesis, by combining a number of chemical building blocks (i.e., reagents such as amino acids). As an example, a linear combinatorial library, e.g., a polypeptide or peptide library, is formed by combining a set of chemical building blocks in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide or peptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

The preparation and screening of combinatorial chemical libraries is well known to those having skill in the pertinent art. Combinatorial libraries include, without limitation, peptide libraries (e.g. U.S. Patent No. 5,010,175; Furka, 1991, *Int. J. Pept. Prot. Res.*, 37:487-493; and Houghton et al., 1991, *Nature*, 354:84-88). Other chemistries for generating chemical diversity libraries can also be used. Nonlimiting examples of chemical diversity library chemistries include, peptides (PCT Publication No. WO 91/019735), encoded peptides (PCT Publication No. WO 93/20242), random bio-oligomers (PCT Publication No. WO 92/00091), benzodiazepines (U.S. Patent No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs et al., 1993, *Proc. Natl. Acad. Sci. USA*, 90:6909-6913), vinyllogous polypeptides (Hagihara et al., 1992, *J. Amer. Chem. Soc.*, 114:6568), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann et al., 1992, *J. Amer. Chem.*

*Soc.*, 114:9217-9218), analogous organic synthesis of small compound libraries (Chen et al., 1994, *J. Amer. Chem. Soc.*, 116:2661), oligocarbamates (Cho et al., 1993, *Science*, 261:1303), and/or peptidyl phosphonates (Campbell et al., 1994, *J. Org. Chem.*, 59:658), nucleic acid libraries (see Ausubel, Berger and Sambrook, all supra),  
5 peptide nucleic acid libraries (U.S. Patent No. 5,539,083), antibody libraries (e.g., Vaughn et al., 1996, *Nature Biotechnology*, 14(3):309-314) and PCT/US96/10287), carbohydrate libraries (e.g., Liang et al., 1996, *Science*, 274:1520-1522) and U.S. Patent No. 5,593,853), small organic molecule libraries (e.g., benzodiazepines, Baum C&EN, Jan. 18, 1993, page 33; and U.S. Patent No. 5,288,514; isoprenoids, U.S.  
10 Patent No. 5,569,588; thiazolidinones and metathiazanones, U.S. Patent No. 5,549,974; pyrrolidines, U.S. Patent Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent No. 5,506,337; and the like).

Devices for the preparation of combinatorial libraries are commercially available (e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY;  
15 Symphony, Rainin, Woburn, MA; 433A Applied Biosystems, Foster City, CA; 9050 Plus, Millipore, Bedford, MA). In addition, a large number of combinatorial libraries are commercially available (e.g., ComGenex, Princeton, NJ; Asinex, Moscow, Russia; Tripos, Inc., St. Louis, MO; ChemStar, Ltd., Moscow, Russia; 3D Pharmaceuticals, Exton, PA; Martek Biosciences, Columbia, MD, and the like).

20 In one embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the cell or tissue expressing an ion channel is attached to a solid phase substrate. In such high throughput assays, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to perform a separate assay  
25 against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 96 modulators. If 1536 well plates are used, then a single plate can easily assay from about 100 to about 1500 different compounds. It is possible to assay several different plates per day; thus, for example,  
30 assay screens for up to about 6,000-20,000 different compounds are possible using the described integrated systems.

In another of its aspects, the present invention encompasses screening and small molecule (e.g., drug) detection assays which involve the detection or identification of small molecules that can bind to a given protein, i.e., a BGS-42 polypeptide or peptide. Particularly preferred are assays suitable for high throughput  
5 screening methodologies.

In such binding-based detection, identification, or screening assays, a functional assay is not typically required. All that is needed is a target protein, preferably substantially purified, and a library or panel of compounds (e.g., ligands, drugs, small molecules) or biological entities to be screened or assayed for binding to  
10 the protein target. Preferably, most small molecules that bind to the target protein will modulate activity in some manner, due to preferential, higher affinity binding to functional areas or sites on the protein.

An example of such an assay is the fluorescence based thermal shift assay (3-Dimensional Pharmaceuticals, Inc., 3DP, Exton, PA) as described in U.S. Patent Nos.  
15 6,020,141 and 6,036,920 to Pantoliano et al.; see also, J. Zimmerman, 2000, *Gen. Eng. News*, 20(8)). The assay allows the detection of small molecules (e.g., drugs, ligands) that bind to expressed, and preferably purified, ion channel polypeptide based on affinity of binding determinations by analyzing thermal unfolding curves of protein-drug or ligand complexes. The drugs or binding molecules determined by this  
20 technique can be further assayed, if desired, by methods, such as those described herein, to determine if the molecules affect or modulate function or activity of the target protein.

To purify a BGS-42 polypeptide or peptide to measure a biological binding or ligand binding activity, the source may be a whole cell lysate that can be prepared by  
25 successive freeze-thaw cycles (e.g., one to three) in the presence of standard protease inhibitors. The BGS-42 polypeptide may be partially or completely purified by standard protein purification methods, e.g., affinity chromatography using specific antibody described *infra*, or by ligands specific for an epitope tag engineered into the recombinant BGS-42 polypeptide molecule, also as described herein. Binding activity  
30 can then be measured as described.

Compounds which are identified according to the methods provided herein, and which modulate or regulate the biological activity or physiology of the BGS-42

polypeptides according to the present invention are a preferred embodiment of this invention. It is contemplated that such modulatory compounds may be employed in treatment and therapeutic methods for treating a condition that is mediated by the novel BGS-42 polypeptides by administering to an individual in need of such treatment a therapeutically effective amount of the compound identified by the methods described herein.

In addition, the present invention provides methods for treating an individual in need of such treatment for a disease, disorder, or condition that is mediated by the BGS-42 polypeptides of the invention, comprising administering to the individual a therapeutically effective amount of the BGS-42-modulating compound identified by a method provided herein.

#### Antisense And Ribozyme (Antagonists)

In specific embodiments, antagonists according to the present invention are nucleic acids corresponding to the sequences contained in SEQ ID NO:1, or the complementary strand thereof, and/or to nucleotide sequences contained a deposited clone. In one embodiment, antisense sequence is generated internally by the organism, in another embodiment, the antisense sequence is separately administered (see, for example, O'Connor, Neurochem., 56:560 (1991). Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Antisense technology can be used to control gene expression through antisense DNA or RNA, or through triple-helix formation. Antisense techniques are discussed for example, in Okano, Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance, Lee et al., Nucleic Acids Research, 6:3073 (1979); Cooney et al., Science, 241:456 (1988); and Dervan et al., Science, 251:1300 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the use of c-myc and c-myb antisense RNA constructs to inhibit the growth of the non-lymphocytic leukemia cell line HL-60 and other cell lines was previously described. (Wickstrom et al. (1988); Anfossi et al. (1989)). These experiments were performed in vitro by incubating cells with the oligoribonucleotide. A similar procedure for in vivo use is described in WO 91/15580. Briefly, a pair of oligonucleotides for a given antisense RNA is produced as follows: A sequence

complimentary to the first 15 bases of the open reading frame is flanked by an EcoR1 site on the 5' end and a HindIII site on the 3' end. Next, the pair of oligonucleotides is heated at 90°C for one minute and then annealed in 2X ligation buffer (20mM TRIS HCl pH 7.5, 10mM MgCl<sub>2</sub>, 10mM dithiothreitol (DTT) and 0.2 mM ATP) and then  
5 ligated to the EcoR1/Hind III site of the retroviral vector PMV7 (WO 91/15580).

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription  
10 thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into receptor polypeptide. Antisense oligonucleotides may be single or double stranded. Double stranded RNA's may be designed based upon the teachings of Paddison et al., Proc. Nat. Acad. Sci., 99:1443-1448 (2002); and  
15 International Publication Nos. WO 01/29058, and WO 99/32619; which are hereby incorporated herein by reference.

In one embodiment, the antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of the  
20 invention. Such a vector would contain a sequence encoding the antisense nucleic acid of the invention. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for  
25 replication and expression in vertebrate cells. Expression of the sequence encoding a polypeptide of the invention, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, Nature, 29:304-310 (1981), the promoter contained in  
30 the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., Cell, 22:787-797 (1980), the herpes thymidine promoter (Wagner et al., Proc. Natl. Acad. Sci. U.S.A.,

78:1441-1445 (1981), the regulatory sequences of the metallothionein gene (Brinster et al., Nature, 296:39-42 (1982)), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a gene of interest. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA" referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded antisense nucleic acids of the invention, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with a RNA sequence of the invention it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. See generally, Wagner, R., Nature, 372:333-335 (1994). Thus, oligonucleotides complementary to either the 5' - or 3' - non-translated, non-coding regions of a polynucleotide sequence of the invention could be used in an antisense approach to inhibit translation of endogenous mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5' -, 3' - or coding region of mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

The polynucleotides of the invention can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556 (1989); Lemaitre et al., Proc. Natl. Acad. Sci., 84:648-652 (1987); PCT Publication NO: WO88/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication NO: WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol et al., BioTechniques, 6:958-976 (1988)) or intercalating agents. (See, e.g., Zon, Pharm. Res., 5:539-549 (1988)). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.



In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group including, but not limited to, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl  
5 phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an a-anomeric oligonucleotide. An a-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual b-units, the strands run parallel to each other (Gautier et al., Nucl. Acids Res., 15:6625-6641 (1987)). The  
10 oligonucleotide is a 2-O-methylribonucleotide (Inoue et al., Nucl. Acids Res., 15:6131-6148 (1987)), or a chimeric RNA-DNA analogue (Inoue et al., FEBS Lett. 215:327-330 (1987)).

Polynucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are  
15 commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (Nucl. Acids Res., 16:3209 (1988)), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., Proc. Natl. Acad. Sci. U.S.A., 85:7448-7451 (1988)), etc.

20 While antisense nucleotides complementary to the coding region sequence of the invention could be used, those complementary to the transcribed untranslated region are most preferred.

Potential antagonists according to the invention also include catalytic RNA, or a ribozyme (See, e.g., PCT International Publication WO 90/11364, published  
25 October 4, 1990; Sarver et al, Science, 247:1222-1225 (1990). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy mRNAs corresponding to the polynucleotides of the invention, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The  
30 sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, Nature, 334:585-591

(1988). There are numerous potential hammerhead ribozyme cleavage sites within each nucleotide sequence disclosed in the sequence listing. Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the mRNA corresponding to the polynucleotides of the invention; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

As in the antisense approach, the ribozymes of the invention can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express the polynucleotides of the invention in vivo. DNA constructs encoding the ribozyme may be introduced into the cell in the same manner as described above for the introduction of antisense encoding DNA. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive promoter, such as, for example, pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous messages and inhibit translation. Since ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Antagonist/agonist compounds may be employed to inhibit the cell growth and proliferation effects of the polypeptides of the present invention on neoplastic cells and tissues, i.e. stimulation of angiogenesis of tumors, and, therefore, retard or prevent abnormal cellular growth and proliferation, for example, in tumor formation or growth.

The antagonist/agonist may also be employed to prevent hyper-vascular diseases, and prevent the proliferation of epithelial lens cells after extracapsular cataract surgery. Prevention of the mitogenic activity of the polypeptides of the present invention may also be desirable in cases such as restenosis after balloon angioplasty.

The antagonist/agonist may also be employed to prevent the growth of scar tissue during wound healing.

The antagonist/agonist may also be employed to treat, prevent, and/or diagnose the diseases described herein.

Thus, the invention provides a method of treating or preventing diseases, disorders, and/or conditions, including but not limited to the diseases, disorders, and/or conditions listed throughout this application, associated with overexpression of a polynucleotide of the present invention by administering to a patient (a) an antisense  
5 molecule directed to the polynucleotide of the present invention, and/or (b) a ribozyme directed to the polynucleotide of the present invention.

#### Biotic Associations

A polynucleotide or polypeptide and/or agonist or antagonist of the present invention may increase the organisms ability, either directly or indirectly, to initiate  
10 and/or maintain biotic associations with other organisms. Such associations may be symbiotic, nonsymbiotic, endosymbiotic, macrosymbiotic, and/or microsymbiotic in nature. In general, a polynucleotide or polypeptide and/or agonist or antagonist of the present invention may increase the organisms ability to form biotic associations with any member of the fungal, bacterial, lichen, mycorrhizal, cyanobacterial,  
15 dinoflagellate, and/or algal, kingdom, phylums, families, classes, genuses, and/or species.

The mechanism by which a polynucleotide or polypeptide and/or agonist or antagonist of the present invention may increase the host organisms ability, either directly or indirectly, to initiate and/or maintain biotic associations is variable, though  
20 may include, modulating osmolarity to desirable levels for the symbiont, modulating pH to desirable levels for the symbiont, modulating secretions of organic acids, modulating the secretion of specific proteins, phenolic compounds, nutrients, or the increased expression of a protein required for host-biotic organisms interactions (e.g., a receptor, ligand, etc.). Additional mechanisms are known in the art and are  
25 encompassed by the invention (see, for example, "Microbial Signalling and Communication", eds., R. England, G. Hobbs, N. Bainton, and D. McL. Roberts, Cambridge University Press, Cambridge, (1999); which is hereby incorporated herein by reference).

In an alternative embodiment, a polynucleotide or polypeptide and/or agonist  
30 or antagonist of the present invention may decrease the host organisms ability to form biotic associations with another organism, either directly or indirectly. The mechanism by which a polynucleotide or polypeptide and/or agonist or antagonist of

the present invention may decrease the host organisms ability, either directly or indirectly, to initiate and/or maintain biotic associations with another organism is variable, though may include, modulating osmolarity to undesirable levels, modulating pH to undesirable levels, modulating secretions of organic acids, modulating the secretion of specific proteins, phenolic compounds, nutrients, or the decreased expression of a protein required for host-biotic organisms interactions (e.g., a receptor, ligand, etc.). Additional mechanisms are known in the art and are encompassed by the invention (see, for example, "Microbial Signalling and Communication", eds., R. England, G. Hobbs, N. Bainton, and D. McL. Roberts, Cambridge University Press, Cambridge, (1999); which is hereby incorporated herein by reference).

The hosts ability to maintain biotic associations with a particular pathogen has significant implications for the overall health and fitness of the host. For example, human hosts have symbiosis with enteric bacteria in their gastrointestinal tracts, particularly in the small and large intestine. In fact, bacteria counts in feces of the distal colon often approach  $10^{12}$  per milliliter of feces. Examples of bowel flora in the gastrointestinal tract are members of the Enterobacteriaceae, Bacteriodes, in addition to a-hemolytic streptococci, E. coli, Bifobacteria, Anaerobic cocci, Eubacteria, Costridia, lactobacilli, and yeasts. Such bacteria, among other things, assist the host in the assimilation of nutrients by breaking down food stuffs not typically broken down by the hosts digestive system, particularly in the hosts bowel. Therefore, increasing the hosts ability to maintain such a biotic association would help assure proper nutrition for the host.

Aberrations in the enteric bacterial population of mammals, particularly humans, has been associated with the following disorders: diarrhea, ileus, chronic inflammatory disease, bowel obstruction, duodenal diverticula, biliary calculous disease, and malnutrition. A polynucleotide or polypeptide and/or agonist or antagonist of the present invention are useful for treating, detecting, diagnosing, prognosing, and/or ameliorating, either directly or indirectly, and of the above mentioned diseases and/or disorders associated with aberrant enteric flora population.

The composition of the intestinal flora, for example, is based upon a variety of factors, which include, but are not limited to, the age, race, diet, malnutrition, gastric

acidity, bile salt excretion, gut motility, and immune mechanisms. As a result, the polynucleotides and polypeptides, including agonists, antagonists, and fragments thereof, may modulate the ability of a host to form biotic associations by affecting, directly or indirectly, at least one or more of these factors.

5           Although the predominate intestinal flora comprises anaerobic organisms, an underlying percentage represents aerobes (e.g., *E. coli*). This is significant as such aerobes rapidly become the predominate organisms in intraabdominal infections – effectively becoming opportunistic early in infection pathogenesis. As a result, there is an intrinsic need to control aerobe populations, particularly for immune  
10           compromised individuals.

          In a preferred embodiment, a polynucleotides and polypeptides, including agonists, antagonists, and fragments thereof, are useful for inhibiting biotic associations with specific enteric symbiont organisms in an effort to control the population of such organisms.

15           Biotic associations occur not only in the gastrointestinal tract, but also on an in the integument. As opposed to the gastrointestinal flora, the cutaneous flora is comprised almost equally with aerobic and anaerobic organisms. Examples of cutaneous flora are members of the gram-positive cocci (e.g., *S. aureus*, coagulase-negative staphylococci, micrococcus, *M. sedentarius*), gram-positive bacilli (e.g.,  
20           *Corynebacterium* species, *C. minutissimum*, *Brevibacterium* species, *Propionibacterium* species, *P. acnes*), gram-negative bacilli (e.g., *Acinetobacter* species), and fungi (*Pityrosporum orbiculare*). The relatively low number of flora associated with the integument is based upon the inability of many organisms to adhere to the skin. The organisms referenced above have acquired this unique ability.  
25           Therefore, the polynucleotides and polypeptides of the present invention may have uses which include modulating the population of the cutaneous flora, either directly or indirectly.

          Aberrations in the cutaneous flora are associated with a number of significant diseases and/or disorders, which include, but are not limited to the following:  
30           impetigo, ecthyma, blistering distal dactylitis, pustules, folliculitis, cutaneous abscesses, pitted keratolysis, trichomycosis axillaris, dermatophytosis complex, axillary odor, erythrasma, cheesy foot odor, acne, tinea versicolor, seborrheic

dermatitis, and *Pityrosporum folliculitis*, to name a few. A polynucleotide or polypeptide and/or agonist or antagonist of the present invention are useful for treating, detecting, diagnosing, prognosing, and/or ameliorating, either directly or indirectly, and of the above mentioned diseases and/or disorders associated with aberrant cutaneous flora population.

Additional biotic associations, including diseases and disorders associated with the aberrant growth of such associations, are known in the art and are encompassed by the invention. See, for example, "Infectious Disease", Second Edition, Eds., S.L., Gorbach, J.G., Bartlett, and N.R., Blacklow, W.B. Saunders Company, Philadelphia, (1998); which is hereby incorporated herein by reference).

#### Pheromones

In another embodiment, a polynucleotide or polypeptide and/or agonist or antagonist of the present invention may increase the organisms ability to synthesize and/or release a pheromone. Such a pheromone may, for example, alter the organisms behavior and/or metabolism.

A polynucleotide or polypeptide and/or agonist or antagonist of the present invention may modulate the biosynthesis and/or release of pheromones, the organisms ability to respond to pheromones (e.g., behaviorally, and/or metabolically), and/or the organisms ability to detect pheromones. Preferably, any of the pheromones, and/or volatiles released from the organism, or induced, by a polynucleotide or polypeptide and/or agonist or antagonist of the invention have behavioral effects the organism.

#### Other Activities

The polypeptide of the present invention, as a result of the ability to stimulate vascular endothelial cell growth, may be employed in treatment for stimulating re-vascularization of ischemic tissues due to various disease conditions such as thrombosis, arteriosclerosis, and other cardiovascular conditions. These polypeptide may also be employed to stimulate angiogenesis and limb regeneration, as discussed above.

The polypeptide may also be employed for treating wounds due to injuries, burns, post-operative tissue repair, and ulcers since they are mitogenic to various cells of different origins, such as fibroblast cells and skeletal muscle cells, and therefore, facilitate the repair or replacement of damaged or diseased tissue.

The polypeptide of the present invention may also be employed stimulate neuronal growth and to treat, prevent, and/or diagnose neuronal damage which occurs in certain neuronal disorders or neuro-degenerative conditions such as Alzheimer's disease, Parkinson's disease, and AIDS-related complex. The polypeptide of the invention may have the ability to stimulate chondrocyte growth, therefore, they may be employed to enhance bone and periodontal regeneration and aid in tissue transplants or bone grafts.

The polypeptides of the present invention may be employed to stimulate growth and differentiation of hematopoietic cells and bone marrow cells when used in combination with other cytokines.

The polypeptide of the invention may also be employed to maintain organs before transplantation or for supporting cell culture of primary tissues.

The polypeptide of the present invention may also be employed for inducing tissue of mesodermal origin to differentiate in early embryos.

The polypeptide or polynucleotides and/or agonist or antagonists of the present invention may also increase or decrease the differentiation or proliferation of embryonic stem cells, besides, as discussed above, hematopoietic lineage.

The polypeptide or polynucleotides and/or agonist or antagonists of the present invention may also be used to modulate mammalian characteristics, such as body height, weight, hair color, eye color, skin, percentage of adipose tissue, pigmentation, size, and shape (e.g., cosmetic surgery). Similarly, polypeptides or polynucleotides and/or agonist or antagonists of the present invention may be used to modulate mammalian metabolism affecting catabolism, anabolism, processing, utilization, and storage of energy.

Polypeptide or polynucleotides and/or agonist or antagonists of the present invention may be used to change a mammal's mental state or physical state by influencing biorhythms, cardiac rhythms, depression (including depressive diseases, disorders, and/or conditions), tendency for violence, tolerance for pain, reproductive capabilities (preferably by Activin or Inhibin-like activity), hormonal or endocrine levels, appetite, libido, memory, stress, or other cognitive qualities.

Polypeptide or polynucleotides and/or agonist or antagonists of the present invention may also be used as a food additive or preservative, such as to increase or

decrease storage capabilities, fat content, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional components.

Polypeptide or polynucleotides and/or agonist or antagonists of the present invention may also be used to increase the efficacy of a pharmaceutical composition, either directly or indirectly. Such a use may be administered in simultaneous conjunction with said pharmaceutical, or separately through either the same or different route of administration (e.g., intravenous for the polynucleotide or polypeptide of the present invention, and orally for the pharmaceutical, among others described herein.).

Polypeptide or polynucleotides and/or agonist or antagonists of the present invention may also be used to prepare individuals for extraterrestrial travel, low gravity environments, prolonged exposure to extraterrestrial radiation levels, low oxygen levels, reduction of metabolic activity, exposure to extraterrestrial pathogens, etc. Such a use may be administered either prior to an extraterrestrial event, during an extraterrestrial event, or both. Moreover, such a use may result in a number of beneficial changes in the recipient, such as, for example, any one of the following, non-limiting, effects: an increased level of hematopoietic cells, particularly red blood cells which would aid the recipient in coping with low oxygen levels; an increased level of B-cells, T-cells, antigen presenting cells, and/or macrophages, which would aid the recipient in coping with exposure to extraterrestrial pathogens, for example; a temporary (i.e., reversible) inhibition of hematopoietic cell production which would aid the recipient in coping with exposure to extraterrestrial radiation levels; increase and/or stability of bone mass which would aid the recipient in coping with low gravity environments; and/or decreased metabolism which would effectively facilitate the recipients ability to prolong their extraterrestrial travel by any one of the following, non-limiting means: (i) aid the recipient by decreasing their basal daily energy requirements; (ii) effectively lower the level of oxidative and/or metabolic stress in recipient (i.e., to enable recipient to cope with increased extraterrestrial radiation levels by decreasing the level of internal oxidative/metabolic damage acquired during normal basal energy requirements; and/or (iii) enabling recipient to subsist at a lower metabolic temperature (i.e., cryogenic, and/or sub-cryogenic environment).



Also preferred is a method of treatment of an individual in need of an increased level of a protein activity, which method comprises administering to such an individual a pharmaceutical composition comprising an amount of an isolated polypeptide, polynucleotide, or antibody of the claimed invention effective to increase  
 5 the level of said protein activity in said individual.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

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## EXAMPLES

## Description of the Preferred Embodiments

## EXAMPLE 1 – BIOINFORMATICS ANALYSIS

5           Tubulin tyrosine ligase domain sequences from several different species were used as probes to search the human genomic sequence database. The search program used was gapped BLAST (Altschul, SF, et al. Nucleic Acids Res 25:3389-3402, 1997.). The top genomic exon hits from the BLAST results were searched back against the non-redundant protein and patent sequence databases. From this analysis, 10 exons encoding potential novel tubulin tyrosine ligases were identified based on sequence homology and their most similar protein sequence were then used as a template to predict more exons using the GENEWISEDB program (Birney and Durbin, 2000). The final predicted exons were assembled and full-length clones of genes were obtained using the predicted exon sequences. With these analyses, a 15 predicted full-length sequence of a novel human secreted protein, named BGS-42 was identified directly from a piece of human genomic sequence (Genbank accession number: AL022327). The complete polynucleotide (SEQ ID NO:1) and polypeptide (SEQ ID NO:2) sequences of BGS-42 are shown Figures 1A-C. Mapping BGS-42 cDNA to human genome found that BGS-42 is located at chromosome 22q13.33 and 20 there are three predicted CpG islands 2.0 kb upstream of its first methionine (Figure 7A-B), indicating BGS-42 has a typical transcription signature.

          BGS-42 was then analyzed for protein domains in Pfam database (Bateman et. al., 2000). The Pfam is a database of multiple alignments of protein domains or conserved protein regions. The alignments provide insight into protein families 25 evolutionary conserved structure, which often has implications for the protein's function. Such Pfams can be very useful for automatically recognizing that a new protein belongs to an existing protein family, even if the homology is weak (A. Bateman, E. Birney, R. Durbin, S.R. Eddy, K.L. Howe, and E.L.L. Sonnhammer. *The Pfam Protein Families Database. Nucleic Acids Research*, 28:263-266, 2000).

30           Based upon the Pfam analysis, the BGS-42 polypeptide was found to have significant sequence homology with TTL domains (amino acids 73 to 365 of SEQ ID NO:2) as shown in Figure 4. Based on sequence, structure and known TTL signature

sequences, this novel protein, BGS-42, is a novel human tubulin tyrosine ligase protein.

## EXAMPLE 2 – METHOD OF CONSTRUCTING A SIZE FRACTIONATED

### 5 BRAIN AND TESTIS cDNA LIBRARY

Poly A<sup>+</sup> RNA from Clontech is treated with DNase I to remove genomic DNA contamination. The RNA is converted into double stranded cDNA using the SuperScript<sup>™</sup> Plasmid System for cDNA Synthesis and Plasmid Cloning (Life Technologies). The cDNA is size fractionated on a TransGenomics HPLC size  
10 exclusion column (TosoHass) with dimensions of 7.8mm x 30cm and a particle size of 10µm. Tris buffered saline is used as the mobile phase, and the column is run at a flow rate of 0.5 ml/min. The system is calibrated using a 1 kb ladder to determine which fractions are to be pooled to obtain the largest cDNA library. Generally, fractions that eluted in the range of 12 to 15 minutes are used. The cDNA is  
15 precipitated, concentrated and then ligated into the Sal I / Not I sites in pSPORT. Following electroporation of the cDNA into DH12S, DNA from the resulting colonies is prepared and subjected to Sal I/Not I restriction enzyme digestion. Generally, the average insert size of libraries made by this procedure is greater than 3.5 Kb and the overall complexity of the library is greater than 10<sup>7</sup> independent clones. The library is  
20 amplified in semi-solid agar for 2 days at 30 C. An aliquot (200 microliters) of the amplified library is inoculated into a 200 ml culture for single-stranded DNA isolation by super-infection with a fi helper phage. The single-stranded circular DNA is concentrated by ethanol precipitation, resuspended at a concentration of one microgram per microliter and used for the cDNA capture experiments.

25

## EXAMPLE 3 – METHOD OF CONVERTING DOUBLE STRANDED cDNA LIBRARIES INTO SINGLE STRAND CIRCULAR FORMS

### I. Preparation of culture.

LB medium (200 mL+ 400 ul carb) is inoculated with 0.2 to 1 ml of thawed  
30 cDNA library. The culture is incubated, shaking at 250 rpm at 37°C for 45 min. The optical density of the culture is measured. The OD600 is preferably between 0.025 and 0.040. One mL M13K07 helper phage is added to the culture and grown for 2

hours. At that time, 500 uL Kanamycin (30 mg/mL) is added and incubation continued for 15-18 hours.

II. Preparation of cells for precipitation.

5 Cultures are poured into six 50 mL tubes. Cells are centrifuged at 10000 rpm in an HB-6 rotor for 15 minutes at 4°C. The supernatant is retrieved and cells discarded. The supernatant is filtered through a 0.2 um filter. DNase I (12000 units from Gibco) is added and incubated at room temperature for 90 minutes.

III. PEG precipitation of DNA.

10 Fifty mL of ice-cold 40% PEG 8000, 2.5 M NaCl, 10 mM MgSO<sub>4</sub> is added to the cell pellets. The solution is mixed and distributed into 6 centrifuge tubes and covered with parafilm. The tubes are incubated on wet ice for 1 hour (or at 4°C overnight).

15 Phage are pelleted at 10000 rpm in an HB-6 rotor for 20 minutes at 4°C. The supernatant is discarded and the sides of the tubes wiped dry. The pellets are resuspended in 1mL TE, pH 8.

The resuspended pellets are placed in a 14 mL Sarstedt tube (6 mL total). SDS is added to 0.1% (60 uL of stock 10% SDS). Proteinase K (60 uL of 20 mg/mL) is then added and incubated at 42C for 1 hour.

20 DNA is extracted with phenol/chloroform by first adding 1 mL of 5M NaCl followed by an equal volume of phenol/chloroform (6 mL). The mixture is vortexed and centrifuged at 5K in an HB-6 rotor for 5 minutes at 4°C. The aqueous (top) phase is transferred to a new Sarstedt tube. Extractions are repeated until no interface is visible.

25 The DNA is precipitated in ethanol by adding 2 volumes of 100% ethanol and precipitating overnight at -20°C. The DNA is centrifuged at 10000 rpm in HB-6 rotor for 20 minutes at 4°C. The ethanol is discarded and the pellets resuspended in 700 uL 70% ethanol. The resuspended pellets are centrifuged at 14000 rpm for 10 minutes at 4°C. The ethanol is discarded and the pellets dried by vacuum.

30 Oligosaccharides are then removed by resuspending the pellet in 50 uL TE, pH 8. The solutions are frozen on dry ice for 10 minutes and centrifuged at 14000rpm for 15 minutes at 4°C. The supernatant is transferred to a new tube and the volume recorded.

The concentration of DNA is determined by measuring absorbance at 260/280. DNA is diluted 1:100 in a quartz cuvette (3 uL DNA + 297 uL TE). The following equation is used to calculate DNA concentration:

$$(32 \text{ ug/mL} \cdot \text{OD})(\text{mL}/100 \text{ uL})(100)(\text{OD}_{260}) = \text{DNA concentration}$$

5 The preferred purity ratio is 1.7 - 2.0.

The DNA is diluted to 1 ug/uL with TB, pH 8 and stored at 4°C.

To test the quality of single-stranded DNA (ssDNA) the following reaction mixtures are prepared:

1. DNA mix per reaction
  - 10 a. 1 uL of 5 ng/uL ssDNA (1:200 dilution of VI.D.2 above)
  - b. 11 uL dH<sub>2</sub>O
  - c. 1.5 uL 10 uM T7 SPORT primer (fresh dilution of stock)
  - d. 1.5 uL 10X Precision-Taq buffer
2. Repair mix per reaction
  - 15 a. 4 uL 5 mM dNTPs (1.25 mM each)
  - b. 1.5 uL 10X Precision-Taq buffer
  - c. 9.25 uL dH<sub>2</sub>O
  - d. 0.25 uL Precision-Taq polymerase
  - e. Preheat cocktail at 70°C until middle of thermal cycle

20 The DNA mixes are aliquoted into PCR tubes and thermal cycle carried out as follows:

1. 95°C, 20 sec
2. 59°C, 1 min; add 15 uL repair mix
3. 73°C, 23 min

25 Ethanol precipitation of the ssDNA is performed by adding 15 ug glycogen, 16 uL 7.5 M NH<sub>4</sub>OAc, 125 uL 100% ethanol. The sample is centrifuged at 14000 rpm for 30 minutes at 4°C and the pellet washed with 125 uL 70% ethanol. The ethanol is discarded and pellet dried by vacuum. The pellet is resuspended in 10 uL TB, pH 8.

30 The DNA is electroporated into DH10B or DH12S cells. A DNA mixture consisting of:

1. 2 uL repaired library (=1.0 x 10<sup>-3</sup> ug)
2. 1 uL 1 ng/uL unrepaired library (= 1.0 x 10<sup>-3</sup> ug)

3. 1 uL 0.01 ug/uL pUC19 positive control DNA (=  $1 \times 10^{-5}$  ug)  
is aliquoted to Eppendorf tubes. Cells are thawed on ice-water. Forty uL of cells are added to each DNA aliquot by pipetting into a chilled cuvette placed between metal plates. Electroporation is carried out at 1.8 kV. Immediately following  
5 electroporation, 1 mL SOC (SOB + glucose +  $Mg^{++}$ ) media is added to the cuvette, then transferred to a 15 mL tube. Cells are allowed to recover for 1 hr at 37°C with shaking (225 rpm). Cells are then plated according to the following dilution scheme:

A. Dilutions of Culture

1. Serial dilutions of culture in 1:10 increments (20 uL into 180 uL LB  
10 broth)
2. Repaired dilutions
  - a. 1:100
  - b. 1:1K
  - c. 1:10K
- 15 3. Unrepaired dilutions
  - a. 1:10
  - b. 1:100
4. Positive control dilutions
  - a. 1:10
  - 20 b. 1:100

100 uL of each dilution is plated on small LB+carb plates and incubated at 37°C overnight. Colonies are counted to calculate titer as follows:

1. use smallest countable dilution
2. (# of colonies)(dilution factor)(200 uL/100 uL)(1000 uL/20 uL) = CFUs
- 25 3. CFUs / ug DNA used = CFU/ug

% Background = (unrepaired CFU/ug / repaired CFU/ug) x 100%

#### EXAMPLE 4 – METHOD OF CLONING THE NOVEL HUMAN BGS-42

##### POLYPEPTIDE OF THE PRESENT INVENTION

30 One microliter of anti-sense biotinylated oligos (or sense oligos when annealing to single stranded DNA from pSPORT2 vector), containing one hundred and fifty nanograms of 1 to 50 different 80mer oligo probes, is added to six



microliters (six micrograms) of a mixture of up to 15 single-stranded covalently closed circular cDNA libraries and seven microliters of 100% formamide in a 0.5 ml PCR tube. The sequence of the 80mer oligos used is as follows:

- 5 5'-AGAACCAGATGGTCAGGGGGTTCCAGTCCGTGACGAGGAACCACTGT  
CTGATGTCGAACTTGGTGTACAGATGAGCAGC -3' (SEQ ID NO:52).

The mixture is heated in a thermal cycler to 95°C for 2 min. Fourteen microliters of 2X hybridization buffer (50% formamide, 1.5 M NaCl, 0.04 M NaPO<sub>4</sub>,  
10 pH 7.2, 5 min EDTA, 0.2% SDS) is added to the heated probe/cDNA library mixture and incubated at 42°C for 26 hours. Hybrids between the biotinylated oligo and the circular cDNA are isolated by diluting the hybridization mixture to 220 microliters solution containing 1 M NaCl, 10 mM Tris-HCl pH 7.5, 1mM EDTA, pH 8.0 and  
15 adding 125 microliters of streptavidin magnetic beads. This solution is incubated at 42°C for 60 min, and mixed every 5 min to re-suspend the beads. The beads are separated from the solution with a magnet and washed three times in 200 microliters of 0.1 X SSPE, 0.1% SDS at 45°C.

The single stranded cDNA is released from the biotinylated oligo/streptavidin magnetic bead complex by adding 50 microliters of 0.1 N NaOH and incubating at  
20 room temperature for 10 min. Six microliters of 3 M sodium acetate is added along with 15 micrograms of glycogen and the solution ethanol precipitated with 120 microliters of 100% ethanol. The precipitated DNA is resuspended in 12 microliters of TB (10 mM Tris-HCl, pH 8.0), 1mM EDTA, pH 8.0). The single-stranded cDNA is converted into double-stranded DNA in a thermal cycler by mixing 5 microliters of  
25 the captured DNA with 1.5 microliters of 10 micromolar standard SP6 primer for libraries in pSPORT 1 and 2 and 17 primer for libraries in pCMVSPORT and 1.5 microliters of 10 X PCR buffer.

Sequences of primers used to repair single-stranded circular DNA isolated from the primary selection are as follows:

30

T7Sport5'- TAATACGACTCACTATAGGG -3' (SEQ ID NO:53)

SP6Sport5'- ATTTAGGTGACACTATAG -3' (SEQ ID NO:54)

The mixture is heated to 95°C for 20 seconds and the temperature gradually brought down to 59°C. Fifteen microliters of a repair mix, that was preheated to 70°C is added to the DNA (repair mix contains 4 microliters of 5 mM dNTPs (1.25 mM each), 1.5 microliters of 10X PCR buffer, 9.25 microliters of water, and 0.25 microliters of Taq polymerase). The solution incubation temperature is raised back to 73°C and incubated for 23 min. The repaired DNA is ethanol precipitated and resuspended in 10 microliters of TB. Electroporation is carried out using two microliters DNA per 40 microliters of E. coli DH12S cells. Three hundred and thirty three microliters are plated onto one 150-mm plate of LB agar plus 100 micrograms/milliliter of ampicillin. After overnight incubation at 37°C, the colonies from all plates are harvested by scraping into 10 ml of LB medium + 50 micrograms/milliliter of ampicillin and 2 ml of sterile glycerol.

The second round of selection is initiated by making single-stranded circular DNA from the primary selected library using the method listed above. The purified single-stranded circular DNA is then assayed with gene-specific primers for each of the targeted sequences using standard PCR conditions.

The sequences of the Gene-Specific-Primer (“GSP”) pairs used to identify the various targeted cDNAs in the primary selected single stranded cDNA libraries are as follows:

Left Primer 1: GTGGGTGGTCCAGAAGTACA (SEQ ID NO:55)

Right Primer 1: GAGAAGCGCTGAGTTGAGAA (SEQ ID NO:56)

Left Primer 2: CACCAGGTTCCAGGAGTACC (SEQ ID NO:57)

Right Primer 2: GAATTGATCTCGATCAGCCA (SEQ ID NO:58)

The secondary hybridization is carried out using only those 80mer biotinylated probes whose targeted sequences were positive with the GSPs. The resulting single-stranded circular DNA is converted to double strands using the antisense oligo for each target sequence as the repair primer (the sense primer is used for material captured from pSPORT2 libraries. The resulting double stranded DNA is

electroporated into DH10B and the resulting colonies inoculated into 96 deep well blocks. Following overnight growth, DNA is prepared and sequentially screened for each of the targeted sequences using the GSPs. The DNA is also cut with Sal I and Not I and the inserts sized by agarose gel electrophoresis.

5           Those cDNA clones that were positive by PCR had the inserts sized and three clones (Clone A, B, and C; SEQ ID NO:9, 10, and 11, respectively) were chosen for DNA sequencing. Clones A, B, and C were used to form a contig and thus derive the full-length consensus sequence provided in Figure 6A-D (SEQ ID NO:12).

10           The consensus sequence (SEQ ID NO:12) was then used to design RT-PCR primers to verify the chimeric assembly. The sequences of the primers are:

BGS42-cloning-L AGACTTCCGGCGCACCAT (SEQ ID NO:59)

BGS42-cloning-R GGGAGAGTGGCTGCAACCTG (SEQ ID NO:60)

15           The full-length nucleotide sequence and the encoded polypeptide for BGS-42 is shown in Figures 1A-C (SEQ ID NO:1).

#### EXAMPLE 5 – EXPRESSION PROFILING OF THE NOVEL HUMAN BGS-42 POLYPEPTIDE

20           The following PCR primer pair was used to measure the steady state levels of BGS-42 mRNA by quantitative PCR:

Sense: 5'- GTTCATGACAGCGTCAGATTCTCT -3' (SEQ ID NO:28)

Antisense:5'- GACAAGATGGTGGAAAGACGTATTC -3' (SEQ ID NO:29)

25           Briefly, first strand cDNA was made from commercially available mRNA. The relative amount of cDNA used in each assay was determined by performing a parallel experiment using a primer pair for a gene expressed in equal amounts in all tissues, cyclophilin. The cyclophilin primer pair detected small variations in the amount of cDNA in each sample and these data were used for normalization of the data obtained with the primer pair for this gene. The PCR data was converted into a relative assessment of the difference in transcript abundance amongst the tissues

30

tested and the data is presented in Figure 8. Transcripts corresponding to the novel tubulin tyrosine ligase protein, BGS-42, were expressed predominately in testis. The BGS-42 polypeptide was also expressed significantly in small intestine, stomach, spinal cord, and to a lesser extent, in brain, and liver.

5 Additional expression profiling was performed to compare the expression pattern of BGS-42 amongst various normal and tumor mRNA tissue sources as shown in Figure 9. As shown, transcripts corresponding to BGS-42 showed differential expression predominately in lung compared to tumor lung tissue, with a 25 fold decrease in expression observed in lung tumors relative to normal lung tissue. The  
10 levels of the control transcript, cyclophilin, used in normalization were approximately equal in all samples indicating that the RNA was of good quality. This apparent loss of BGS-42 expression in tumor tissues relative to normal tissues suggests BGS-42 may play a role in tumor suppression, either directly or indirectly. These data also show skeletal muscle as a tissue with relative high abundance.

15

#### EXAMPLE 6 – METHOD OF ASSESSING THE EXPRESSION PROFILE OF THE NOVEL BGS-42 POLYPEPTIDES OF THE PRESENT INVENTION USING EXPANDED mRNA TISSUE AND CELL SOURCES

Total RNA from tissues was isolated using the TriZol protocol (Invitrogen)  
20 and quantified by determining its absorbance at 260nM. An assessment of the 18s and 28s ribosomal RNA bands was made by denaturing gel electrophoresis to determine RNA integrity.

The specific sequence to be measured was aligned with related genes found in GenBank to identify regions of significant sequence divergence to maximize primer  
25 and probe specificity. Gene-specific primers and probes were designed using the ABI primer express software to amplify small amplicons (150 base pairs or less) to maximize the likelihood that the primers function at 100% efficiency. All primer/probe sequences were searched against Public Genbank databases to ensure target specificity. Primers and probes were obtained from ABI.

For BGS-42, the primer probe sequences were as follows:

Forward Primer 5'-TCAGAGAATGGGCCAACAAGA-3' (SEQ ID NO:28)

Reverse Primer 5'-CGAAAACGCTCGAGGAATGA-3' (SEQ ID NO:29)

5 TaqMan Probe 5'-CAGGCCTAGGTTCTCCTCTCGGAAA-3' (SEQ ID NO:30)

#### DNA contamination

To access the level of contaminating genomic DNA in the RNA, the RNA was divided into 2 aliquots and one half was treated with Rnase-free Dnase (Invitrogen).  
10 Samples from both the Dnase-treated and non-treated were then subjected to reverse transcription reactions with (RT+) and without (RT-) the presence of reverse transcriptase. TaqMan assays were carried out with gene-specific primers (see above) and the contribution of genomic DNA to the signal detected was evaluated by comparing the threshold cycles obtained with the RT+/RT- non-Dnase treated RNA to  
15 that on the RT+/RT- Dnase treated RNA. The amount of signal contributed by genomic DNA in the Dnased RT- RNA must be less than 10% of that obtained with Dnased RT+ RNA. If not the RNA was not used in actual experiments.

#### Reverse Transcription reaction and Sequence Detection

100ng of Dnase-treated total RNA was annealed to 2.5  $\mu$ M of the respective  
20 gene-specific reverse primer in the presence of 5.5 mM Magnesium Chloride by heating the sample to 72°C for 2 min and then cooling to 55° C for 30 min. 1.25 U/ $\mu$ l of MuLr reverse transcriptase and 500 $\mu$ M of each dNTP was added to the reaction and the tube was incubated at 37° C for 30 min. The sample was then heated to 90°C for 5 min to denature enzyme.

25 Quantitative sequence detection was carried out on an ABI PRISM 7700 by adding to the reverse transcribed reaction 2.5 $\mu$ M forward and reverse primers, 500 $\mu$ M of each dNTP, buffer and 5U AmpliTaq Gold™. The PCR reaction was then held at 94°C for 12 min, followed by 40 cycles of 94° C for 15 sec and 60° C for 30 sec.

#### Data handling

30 The threshold cycle (Ct) of the lowest expressing tissue (the highest Ct value) was used as the baseline of expression and all other tissues were expressed as the

relative abundance to that tissue by calculating the difference in Ct value between the baseline and the other tissues and using it as the exponent in  $2^{(\Delta C_t)}$

The expanded expression profile of the BGS-42 polypeptide is provided in Figure 10. The BGS-42 polypeptide was expressed predominately in the vas deferens.

5 Expression of BGS-42 was also significantly expressed in lymph gland, pituitary, placenta, and to a lesser extent, in other tissues as shown.

Additional expression profiling was performed to compare the BGS-42 expression in normal versus tumor tissues. The expression of the BGS-42 polypeptide was not observed in any of the five testis tumor samples. This apparent loss of BGS-  
10 42 expression in tumor tissues relative to normal tissues suggests BGS-42 may play a role in tumor suppression, either directly or indirectly.

#### EXAMPLE 7 – METHOD OF ASSESSING THE ABILITY OF BGS-42 TO LIGATE TYROSINE TO TUBULIN

15 The method of Raybin and Flavin may be followed essentially as described in Biochemistry 16, 2189-2194. Briefly, purified BGS42 protein may be tested for tubulin tyrosine ligase activity by incubating with appropriate levels of tubulin and tyrosine and  $^{14}\text{C}$ - tyrosine in the presence of ATP. The reaction may be stopped after an appropriate period of time, and the reaction products precipitated with  
20 trichloroacetic acid and filtering onto Whatman 3MM. The specificity of the reaction may be checked by SDS-polyacrylamide electrophoresis and autoradiography. Incorporation of  $^{14}\text{C}$ - tyrosine onto tubulin would be diagnostic of active tubulin tyrosine ligase activity. The skilled artisan would appreciate that appropriate levels of tubulin, tyrosine, and  $^{14}\text{C}$ - tyrosine would need to be empirically  
25 determined based upon the  $K_{cat}$  and  $K_m$ , and other catalytic rate constants, of the purified BGS-42 polypeptide. The same rate constants would also influence selection of an appropriate incubation period.

One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention, agonists, and/or antagonists of  
30 polynucleotides or polypeptides of the invention.

#### EXAMPLE 8 – METHOD OF DEMONSTRATING ALTERED METHYLATION PATTERNS IN THE BGS42 PROMOTOR

The method of Herman et al., may be followed essentially as described by Herman et al., Proc. Natl. Acad. Sci. USA 93, 9821-9826. Briefly, breast, colon and lung tumor cell lines may be assayed for BGS-42 expression by real time quantitative PCR. Expression levels may be ranked as being either high or low expression. Genomic DNA may be isolated by standard techniques, denatured with sodium hydroxide and treated with sodium bisulfite. Two sets of PCR primers may then be designed across several regions upstream of the start of BGS-42 translation that include CpG islands. To amplify the genomic DNA from high expression cell lines (non-methylated DNA), a T may be used in the position 5' to the G where normally a C would reside. To amplify the genomic DNA isolated from the low expressing cell lines (methylated DNA); the normal C will be used in the position 5' to the G. These primer sets will take advantage of the fact the methylation protects cytosine from conversion to uracil by bisulfite treatment. Using primers with both C and T will distinguish methylated DNA from non-methylated. Thus, the changes in expression levels between the "C" and "T" containing primers will provide evidence that DNA methylation does have an effect on BGS-42 expression, in tumor samples relative to normal samples.

One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention, agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

#### EXAMPLE 9 – METHOD OF ASSESSING THE PHYSIOLOGICAL FUNCTION OF THE BGS-42 POLYPEPTIDE AT THE CELLULAR LEVEL

The physiological function of the BGS-42 polypeptide may be assessed by expressing the sequences encoding BGS-42 at physiologically elevated levels in mammalian cell culture systems. cDNA is subcloned into a mammalian expression vector containing a strong promoter that drives high levels of cDNA expression (examples are provided elsewhere herein). Vectors of choice include pCMV SPORT (Life Technologies) and pCR3.1 (Invitrogen, Carlsbad CA), both of which contain the cytomegalovirus promoter. 5-10, ug of recombinant vector are transiently transfected

into a human cell line, preferably of endothelial or hematopoietic origin, using either liposome formulations or electroporation. 1-2ug of an additional plasmid containing sequences encoding a marker protein are cotransfected. Expression of a marker protein provides a means to distinguish transfected cells from nontransfected cells and is a reliable predictor of cDNA expression from the recombinant vector. Marker proteins of choice include, e.g., Green Fluorescent Protein (GFP; Clontech), CD64, or a CD64-GFP fusion protein. Flow cytometry (FCM), an automated, laser optics-based technique, is used to identify transfected cells expressing GFP or CD64-GFP and to evaluate the apoptotic state of the cells and other cellular properties. FCM detects and quantifies the uptake of fluorescent molecules that diagnose events preceding or coincident with cell death. These events include changes in nuclear DNA content as measured by staining of DNA with propidium iodide; changes in cell size and granularity as measured by forward light scatter and 90 degree side light scatter; down-regulation of DNA synthesis as measured by decrease in bromodeoxyuridine uptake; alterations in expression of cell surface and intracellular proteins as measured by reactivity with specific antibodies; and alterations in plasma membrane composition as measured by the binding of fluorescein-conjugated Annexin V protein to the cell surface. Methods in flow cytometry are discussed in Ormerod, M. G. (1994) *Flow Cytometry*, Oxford, New York NY.

The influence of BGS-42 polypeptides on gene expression can be assessed using highly purified populations of cells transfected with sequences encoding BGS-42 and either CD64 or CD64-GFP. CD64 and CD64-GFP are expressed on the surface of transfected cells and bind to conserved regions of human immunoglobulin G (IgG). Transfected cells are efficiently separated from nontransfected cells using magnetic beads coated with either human IgG or antibody against CD64 (DYNAL, Lake Success NY). mRNA can be purified from the cells using methods well known by those of skill in the art. Expression of mRNA encoding BGS-42 polypeptides and other genes of interest can be analyzed by northern analysis or microarray techniques.



EXAMPLE 10 – METHOD OF SCREENING FOR COMPOUNDS THAT  
INTERACT WITH THE BGS-42 POLYPEPTIDE

The following assays are designed to identify compounds that bind to the BGS-42 polypeptide, bind to other cellular proteins that interact with the BGS-42 polypeptide, and to compounds that interfere with the interaction of the BGS-42 polypeptide with other cellular proteins.

Such compounds can include, but are not limited to, other cellular proteins. Specifically, such compounds can include, but are not limited to, peptides, such as, for example, soluble peptides, including, but not limited to Ig-tailed fusion peptides, comprising extracellular portions of BGS-42 polypeptide transmembrane receptors, and members of random peptide libraries (see, e.g., Lam, K. S. et al., 1991, Nature 354:82-84; Houghton, R. et al., 1991, Nature 354:84-86), made of D-and/or L-configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate phosphopeptide libraries; see, e.g., Songyang, Z., et al., 1993, Cell 72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab').sub.2 and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

Compounds identified via assays such as those described herein can be useful, for example, in elaborating the biological function of the BGS-42 polypeptide, and for ameliorating symptoms of tumor progression, for example. In instances, for example, whereby a tumor progression state or disorder results from a lower overall level of BGS-42 expression, BGS-42 polypeptide, and/or BGS-42 polypeptide activity in a cell involved in the tumor progression state or disorder, compounds that interact with the BGS-42 polypeptide can include ones which accentuate or amplify the activity of the bound BGS-42 polypeptide. Such compounds would bring about an effective increase in the level of BGS-42 polypeptide activity, thus ameliorating symptoms of the tumor progression disorder or state. In instances whereby mutations within the BGS-42 polypeptide cause aberrant BGS-42 polypeptides to be made which have a deleterious effect that leads to tumor progression, compounds that bind BGS-42 polypeptide can be identified that inhibit the activity of the bound BGS-42



unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously immobilized component is pre-labeled, the detection of label  
5 immobilized on the surface indicates that complexes were formed. Where the previously nonimmobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the immobilized component (the antibody, in turn, can be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

10 Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for BGS-42 polypeptide or the test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

15  
**EXAMPLE 12 – METHOD OF IDENTIFYING COMPOUNDS THAT INTERFERE  
WITH BGS-42 POLYPEPTIDE/CELLULAR PRODUCT INTERACTION**

The BGS-42 polypeptide of the invention can, in vivo, interact with one or more cellular or extracellular macromolecules, such as proteins. Such  
20 macromolecules include, but are not limited to, polypeptides, particularly GPCR ligands, and those products identified via screening methods described, elsewhere herein. For the purposes of this discussion, such cellular and extracellular macromolecules are referred to herein as "binding partner(s)". For the purpose of the present invention, "binding partner" may also encompass polypeptides, small  
25 molecule compounds, polysaccharides, lipids, and any other molecule or molecule type referenced herein. Compounds that disrupt such interactions can be useful in regulating the activity of the BGS-42 polypeptide, especially mutant BGS-42 polypeptide. Such compounds can include, but are not limited to molecules such as antibodies, peptides, and the like described in elsewhere herein.

30 The basic principle of the assay systems used to identify compounds that interfere with the interaction between the BGS-42 polypeptide and its cellular or extracellular binding partner or partners involves preparing a reaction mixture

containing the BGS-42 polypeptide, and the binding partner under conditions and for a time sufficient to allow the two products to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound can be initially included in the reaction mixture, or can be added at a time subsequent to the addition of BGS-42 polypeptide and its cellular or extracellular binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the BGS-42 polypeptide and the cellular or extracellular binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the BGS-42 polypeptide and the interactive binding partner. Additionally, complex formation within reaction mixtures containing the test compound and normal BGS-42 polypeptide can also be compared to complex formation within reaction mixtures containing the test compound and mutant BGS-42 polypeptide. This comparison can be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal BGS-42 polypeptide.

The assay for compounds that interfere with the interaction of the BGS-42 polypeptide and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the BGS-42 polypeptide or the binding partner onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the BGS-42 polypeptide and the binding partners, e.g., by competition, can be identified by conducting the reaction in the presence of the test substance; i.e., by adding the test substance to the reaction mixture prior to or simultaneously with the BGS-42 polypeptide and interactive cellular or extracellular binding partner. Alternatively, test compounds that disrupt preformed complexes, e.g. compounds with higher binding constants that displace one of the components from the complex, can be tested by

adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

5 In a heterogeneous assay system, either the BGS-42 polypeptide or the interactive cellular or extracellular binding partner, is anchored onto a solid surface, while the non-anchored species is labeled, either directly or indirectly. In practice, microtitre plates are conveniently utilized. The anchored species can be immobilized by non-covalent or covalent attachments. Non-covalent attachment can be accomplished simply by coating the solid surface with a solution of the BGS-42 polypeptide or binding partner and drying. Alternatively, an immobilized antibody  
10 specific for the species to be anchored can be used to anchor the species to the solid surface. The surfaces can be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g., by washing) and any complexes  
15 formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the initially non-immobilized  
20 species (the antibody, in turn, can be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

25 Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again,  
30 depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt preformed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the BGS-42 polypeptide and the interactive cellular or extracellular binding partner product is prepared in which either the BGS-42 polypeptide or their binding partners are labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Pat. No. 4,109,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances which disrupt BGS-42 polypeptide-cellular or extracellular binding partner interaction can be identified.

In a particular embodiment, the BGS-42 polypeptide can be prepared for immobilization using recombinant DNA techniques known in the art. For example, the BGS-42 polypeptide coding region can be fused to a glutathione-S-transferase (GST) gene using a fusion vector such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion product. The interactive cellular or extracellular product can be purified and used to raise a monoclonal antibody, using methods routinely practiced in the art and described above. This antibody can be labeled with the radioactive isotope <sup>125</sup>I, for example, by methods routinely practiced in the art. In a heterogeneous assay, e.g., the GST- BGS-42 polypeptide fusion product can be anchored to glutathione-agarose beads. The interactive cellular or extracellular binding partner product can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the BGS-42 polypeptide and the interactive cellular or extracellular binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

Alternatively, the GST- BGS-42 polypeptide fusion product and the interactive cellular or extracellular binding partner product can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can

be added either during or after the binding partners are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the binding partner interaction can be detected by adding the labeled antibody and measuring the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of the BGS-42 polypeptide product and the interactive cellular or extracellular binding partner (in case where the binding partner is a product), in place of one or both of the full length products.

Any number of methods routinely practiced in the art can be used to identify and isolate the protein's binding site. These methods include, but are not limited to, mutagenesis of one of the genes encoding one of the products and screening for disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the gene encoding the second species in the complex can be selected. Sequence analysis of the genes encoding the respective products will reveal the mutations that correspond to the region of the product involved in interactive binding. Alternatively, one product can be anchored to a solid surface using methods described in this Section above, and allowed to interact with and bind to its labeled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labeled peptide comprising the binding domain can remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the cellular or extracellular binding partner product is obtained, short gene segments can be engineered to express peptide fragments of the product, which can then be tested for binding activity and purified or synthesized.

#### EXAMPLE 13 – ISOLATION OF A SPECIFIC CLONE FROM THE DEPOSITED SAMPLE

The deposited material in the sample assigned the ATCC Deposit Number cited in Table I for any given cDNA clone also may contain one or more additional plasmids, each comprising a cDNA clone different from that given clone. Thus, deposits sharing the same ATCC Deposit Number contain at least a plasmid for each

cDNA clone identified in Table I. Typically, each ATCC deposit sample cited in Table I comprises a mixture of approximately equal amounts (by weight) of about 1-10 plasmid DNAs, each containing a different cDNA clone and/or partial cDNA clone; but such a deposit sample may include plasmids for more or less than 2 cDNA clones.

Two approaches can be used to isolate a particular clone from the deposited sample of plasmid DNA(s) cited for that clone in Table I. First, a plasmid is directly isolated by screening the clones using a polynucleotide probe corresponding to SEQ ID NO:1.

Particularly, a specific polynucleotide with 30-40 nucleotides is synthesized using an Applied Biosystems DNA synthesizer according to the sequence reported. The oligonucleotide is labeled, for instance, with  $^{32}\text{P}$ -(-ATP using T4 polynucleotide kinase and purified according to routine methods. (E.g., Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring, NY (1982).) The plasmid mixture is transformed into a suitable host, as indicated above (such as XL-1 Blue (Stratagene)) using techniques known to those of skill in the art, such as those provided by the vector supplier or in related publications or patents cited above. The transformants are plated on 1.5% agar plates (containing the appropriate selection agent, e.g., ampicillin) to a density of about 150 transformants (colonies) per plate. These plates are screened using Nylon membranes according to routine methods for bacterial colony screening (e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edit., (1989), Cold Spring Harbor Laboratory Press, pages 1.93 to 1.104), or other techniques known to those of skill in the art.

Alternatively, two primers of 17-20 nucleotides derived from both ends of the SEQ ID NO:1 (i.e., within the region of SEQ ID NO:1 bounded by the 5' NT and the 3' NT of the clone defined in Table I) are synthesized and used to amplify the desired cDNA using the deposited cDNA plasmid as a template. The polymerase chain reaction is carried out under routine conditions, for instance, in 25 ul of reaction mixture with 0.5 ug of the above cDNA template. A convenient reaction mixture is 1.5-5 mM  $\text{MgCl}_2$ , 0.01% (w/v) gelatin, 20 uM each of dATP, dCTP, dGTP, dTTP, 25 pmol of each primer and 0.25 Unit of Taq polymerase. Thirty five cycles of PCR (denaturation at 94 degree C for 1 min; annealing at 55 degree C for 1 min; elongation



at 72 degree C for 1 min) are performed with a Perkin-Elmer Cetus automated thermal cyclor. The amplified product is analyzed by agarose gel electrophoresis and the DNA band with expected molecular weight is excised and purified. The PCR product is verified to be the selected sequence by subcloning and sequencing the DNA product.

5           The polynucleotide(s) of the present invention, the polynucleotide encoding the polypeptide of the present invention, or the polypeptide encoded by the deposited clone may represent partial, or incomplete versions of the complete coding region (i.e., full-length gene). Several methods are known in the art for the identification of the 5' or 3' non-coding and/or coding portions of a gene which may not be present in  
10 the deposited clone. The methods that follow are exemplary and should not be construed as limiting the scope of the invention. These methods include but are not limited to, filter probing, clone enrichment using specific probes, and protocols similar or identical to 5' and 3' "RACE" protocols that are well known in the art. For instance, a method similar to 5' RACE is available for generating the missing 5' end  
15 of a desired full-length transcript. (Fromont-Racine et al., Nucleic Acids Res. 21(7):1683-1684 (1993)).

          Briefly, a specific RNA oligonucleotide is ligated to the 5' ends of a population of RNA presumably containing full-length gene RNA transcripts. A primer set containing a primer specific to the ligated RNA oligonucleotide and a  
20 primer specific to a known sequence of the gene of interest is used to PCR amplify the 5' portion of the desired full-length gene. This amplified product may then be sequenced and used to generate the full-length gene.

          This above method starts with total RNA isolated from the desired source, although poly-A+ RNA can be used. The RNA preparation can then be treated with  
25 phosphatase if necessary to eliminate 5' phosphate groups on degraded or damaged RNA that may interfere with the later RNA ligase step. The phosphatase should then be inactivated and the RNA treated with tobacco acid pyrophosphatase in order to remove the cap structure present at the 5' ends of messenger RNAs. This reaction leaves a 5' phosphate group at the 5' end of the cap cleaved RNA which can then be  
30 ligated to an RNA oligonucleotide using T4 RNA ligase.

          This modified RNA preparation is used as a template for first strand cDNA synthesis using a gene specific oligonucleotide. The first strand synthesis reaction is

used as a template for PCR amplification of the desired 5' end using a primer specific to the ligated RNA oligonucleotide and a primer specific to the known sequence of the gene of interest. The resultant product is then sequenced and analyzed to confirm that the 5' end sequence belongs to the desired gene. Moreover, it may be  
5 advantageous to optimize the RACE protocol to increase the probability of isolating additional 5' or 3' coding or non-coding sequences. Various methods of optimizing a RACE protocol are known in the art, though a detailed description summarizing these methods can be found in B.C. Schaefer, *Anal. Biochem.*, 227:255-273, (1995).

An alternative method for carrying out 5' or 3' RACE for the identification of  
10 coding or non-coding sequences is provided by Frohman, M.A., et al., *Proc.Nat'l.Acad.Sci.USA*, 85:8998-9002 (1988). Briefly, a cDNA clone missing either the 5' or 3' end can be reconstructed to include the absent base pairs extending to the translational start or stop codon, respectively. In some cases, cDNAs are missing the start of translation, therefor. The following briefly describes a modification of this  
15 original 5' RACE procedure. Poly A<sup>+</sup> or total RNAs reverse transcribed with Superscript II (Gibco/BRL) and an antisense or I complementary primer specific to the cDNA sequence. The primer is removed from the reaction with a Microcon Concentrator (Amicon). The first-strand cDNA is then tailed with dATP and terminal deoxynucleotide transferase (Gibco/BRL). Thus, an anchor sequence is produced  
20 which is needed for PCR amplification. The second strand is synthesized from the dA-tail in PCR buffer, Taq DNA polymerase (Perkin-Elmer Cetus), an oligo-dT primer containing three adjacent restriction sites (XhoI, SalI and ClaI) at the 5' end and a primer containing just these restriction sites. This double-stranded cDNA is PCR amplified for 40 cycles with the same primers as well as a nested cDNA-specific  
25 antisense primer. The PCR products are size-separated on an ethidium bromide-agarose gel and the region of gel containing cDNA products the predicted size of missing protein-coding DNA is removed. cDNA is purified from the agarose with the Magic PCR Prep kit (Promega), restriction digested with XhoI or SalI, and ligated to a plasmid such as pBluescript SKII (Stratagene) at XhoI and EcoRV sites. This DNA is  
30 transformed into bacteria and the plasmid clones sequenced to identify the correct protein-coding inserts. Correct 5' ends are confirmed by comparing this sequence with the putatively identified homologue and overlap with the partial cDNA clone. Similar

methods known in the art and/or commercial kits are used to amplify and recover 3' ends.

Several quality-controlled kits are commercially available for purchase. Similar reagents and methods to those above are supplied in kit form from  
5 Gibco/BRL for both 5' and 3' RACE for recovery of full length genes. A second kit is available from Clontech which is a modification of a related technique, SLIC (single-stranded ligation to single-stranded cDNA), developed by Dumas et al., *Nucleic Acids Res.*, 19:5227-32(1991). The major differences in procedure are that the RNA is alkaline hydrolyzed after reverse transcription and RNA ligase is used to join a  
10 restriction site-containing anchor primer to the first-strand cDNA. This obviates the necessity for the dA-tailing reaction which results in a polyT stretch that is difficult to sequence past.

An alternative to generating 5' or 3' cDNA from RNA is to use cDNA library double-stranded DNA. An asymmetric PCR-amplified antisense cDNA strand is  
15 synthesized with an antisense cDNA-specific primer and a plasmid-anchored primer. These primers are removed and a symmetric PCR reaction is performed with a nested cDNA-specific antisense primer and the plasmid-anchored primer.

#### RNA Ligase Protocol For Generating The 5' or 3' End Sequences

##### To Obtain Full Length Genes

20 Once a gene of interest is identified, several methods are available for the identification of the 5' or 3' portions of the gene which may not be present in the original cDNA plasmid. These methods include, but are not limited to, filter probing, clone enrichment using specific probes and protocols similar and identical to 5' and 3'RACE. While the full-length gene may be present in the library and can be  
25 identified by probing, a useful method for generating the 5' or 3' end is to use the existing sequence information from the original cDNA to generate the missing information. A method similar to 5'RACE is available for generating the missing 5' end of a desired full-length gene. (This method was published by Fromont-Racine et al., *Nucleic Acids Res.*, 21(7): 1683-1684 (1993)). Briefly, a specific RNA  
30 oligonucleotide is ligated to the 5' ends of a population of RNA presumably 30 containing full-length gene RNA transcript and a primer set containing a primer specific to the ligated RNA oligonucleotide and a primer specific to a known

sequence of the gene of interest, is used to PCR amplify the 5' portion of the desired full length gene which may then be sequenced and used to generate the full length gene. This method starts with total RNA isolated from the desired source, poly A RNA may be used but is not a prerequisite for this procedure. The RNA preparation  
5 may then be treated with phosphatase if necessary to eliminate 5' phosphate groups on degraded or damaged RNA which may interfere with the later RNA ligase step. The phosphatase if used is then inactivated and the RNA is treated with tobacco acid pyrophosphatase in order to remove the cap structure present at the 5' ends of messenger RNAs. This reaction leaves a 5' phosphate group at the 5' end of the cap  
10 cleaved RNA which can then be ligated to an RNA oligonucleotide using T4 RNA ligase. This modified RNA preparation can then be used as a template for first strand cDNA synthesis using a gene specific oligonucleotide. The first strand synthesis reaction can then be used as a template for PCR amplification of the desired 5' end using a primer specific to the ligated RNA oligonucleotide and a primer specific to the  
15 known sequence of the apoptosis related of interest. The resultant product is then sequenced and analyzed to confirm that the 5' end sequence belongs to the relevant apoptosis related.

#### EXAMPLE 14 – BACTERIAL EXPRESSION OF A POLYPEPTIDE

20 A polynucleotide encoding a polypeptide of the present invention is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' ends of the DNA sequence, as outlined in Example 13, to synthesize insertion fragments. The primers used to amplify the cDNA insert should preferably contain restriction sites, such as BamHI and XbaI, at the 5' end of the primers in order to clone the amplified product  
25 into the expression vector. For example, BamHI and XbaI correspond to the restriction enzyme sites on the bacterial expression vector pQE-9. (Qiagen, Inc., Chatsworth, CA). This plasmid vector encodes antibiotic resistance (Ampr), a bacterial origin of replication (ori), an IPTG-regulatable promoter/operator (P/O), a ribosome binding site (RBS), a 6-histidine tag (6-His), and restriction enzyme cloning  
30 sites.

The pQE-9 vector is digested with BamHI and XbaI and the amplified fragment is ligated into the pQE-9 vector maintaining the reading frame initiated at

the bacterial RBS. The ligation mixture is then used to transform the *E. coli* strain M15/rep4 (Qiagen, Inc.) which contains multiple copies of the plasmid pREP4, that expresses the *lacI* repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants are identified by their ability to grow on LB plates and  
5 ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis.

Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The  
10 cells are grown to an optical density 600 (O.D.600) of between 0.4 and 0.6. IPTG (Isopropyl-B-D-thiogalacto pyranoside) is then added to a final concentration of 1 mM. IPTG induces by inactivating the *lacI* repressor, clearing the P/O leading to increased gene expression.

Cells are grown for an extra 3 to 4 hours. Cells are then harvested by  
15 centrifugation (20 mins at 6000Xg). The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl by stirring for 3-4 hours at 4 degree C. The cell debris is removed by centrifugation, and the supernatant containing the polypeptide is loaded onto a nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin column (available from QIAGEN, Inc., supra). Proteins with a 6 x His tag bind to the Ni-NTA resin with high  
20 affinity and can be purified in a simple one-step procedure (for details see: The QIAexpressionist (1995) QIAGEN, Inc., supra).

Briefly, the supernatant is loaded onto the column in 6 M guanidine-HCl, pH 8, the column is first washed with 10 volumes of 6 M guanidine-HCl, pH 8, then washed with 10 volumes of 6 M guanidine-HCl pH 6, and finally the polypeptide is  
25 eluted with 6 M guanidine-HCl, pH 5.

The purified protein is then renatured by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be successfully refolded while immobilized on the Ni-NTA column. The recommended conditions are as follows: renature using a linear  
30 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20 mM Tris/HCl pH 7.4, containing protease inhibitors. The renaturation should be performed over a period of 1.5 hours or more. After renaturation the proteins are eluted by the addition of 250

mM imidazole. Imidazole is removed by a final dialyzing step against PBS or 50 mM sodium acetate pH 6 buffer plus 200 mM NaCl. The purified protein is stored at 4 degree C or frozen at -80 degree C.

5                                    **EXAMPLE 15 – PURIFICATION OF A POLYPEPTIDE  
FROM AN INCLUSION BODY**

The following alternative method can be used to purify a polypeptide expressed in E coli when it is present in the form of inclusion bodies. Unless otherwise specified, all of the following steps are conducted at 4-10 degree C.

10            Upon completion of the production phase of the E. coli fermentation, the cell culture is cooled to 4-10 degree C and the cells harvested by continuous centrifugation at 15,000 rpm (Heraeus Sepatech). On the basis of the expected yield of protein per unit weight of cell paste and the amount of purified protein required, an appropriate amount of cell paste, by weight, is suspended in a buffer solution  
15            containing 100 mM Tris, 50 mM EDTA, pH 7.4. The cells are dispersed to a homogeneous suspension using a high shear mixer.

The cells are then lysed by passing the solution through a microfluidizer (Microfluidics, Corp. or APV Gaulin, Inc.) twice at 4000-6000 psi. The homogenate is then mixed with NaCl solution to a final concentration of 0.5 M NaCl, followed by  
20            centrifugation at 7000 xg for 15 min. The resultant pellet is washed again using 0.5M NaCl, 100 mM Tris, 50 mM EDTA, pH 7.4.

The resulting washed inclusion bodies are solubilized with 1.5 M guanidine hydrochloride (GuHCl) for 2-4 hours. After 7000 xg centrifugation for 15 min., the pellet is discarded and the polypeptide containing supernatant is incubated at 4 degree  
25            C overnight to allow further GuHCl extraction.

Following high speed centrifugation (30,000 xg) to remove insoluble particles, the GuHCl solubilized protein is refolded by quickly mixing the GuHCl extract with 20 volumes of buffer containing 50 mM sodium, pH 4.5, 150 mM NaCl, 2 mM EDTA by vigorous stirring. The refolded diluted protein solution is kept at 4 degree C  
30            without mixing for 12 hours prior to further purification steps.

To clarify the refolded polypeptide solution, a previously prepared tangential filtration unit equipped with 0.16 um membrane filter with appropriate surface area

(e.g., Filtron), equilibrated with 40 mM sodium acetate, pH 6.0 is employed. The filtered sample is loaded onto a cation exchange resin (e.g., Poros HS-50, Perceptive Biosystems). The column is washed with 40 mM sodium acetate, pH 6.0 and eluted with 250 mM, 500 mM, 1000 mM, and 1500 mM NaCl in the same buffer, in a  
5 stepwise manner. The absorbance at 280 nm of the effluent is continuously monitored. Fractions are collected and further analyzed by SDS-PAGE.

Fractions containing the polypeptide are then pooled and mixed with 4 volumes of water. The diluted sample is then loaded onto a previously prepared set of tandem columns of strong anion (Poros HQ-50, Perceptive Biosystems) and weak  
10 anion (Poros CM-20, Perceptive Biosystems) exchange resins. The columns are equilibrated with 40 mM sodium acetate, pH 6.0. Both columns are washed with 40 mM sodium acetate, pH 6.0, 200 mM NaCl. The CM-20 column is then eluted using a 10 column volume linear gradient ranging from 0.2 M NaCl, 50 mM sodium acetate, pH 6.0 to 1.0 M NaCl, 50 mM sodium acetate, pH 6.5. Fractions are collected under  
15 constant A280 monitoring of the effluent. Fractions containing the polypeptide (determined, for instance, by 16% SDS-PAGE) are then pooled.

The resultant polypeptide should exhibit greater than 95% purity after the above refolding and purification steps. No major contaminant bands should be observed from Coomassie blue stained 16% SDS-PAGE gel when 5 ug of purified  
20 protein is loaded. The purified protein can also be tested for endotoxin/LPS contamination, and typically the LPS content is less than 0.1 ng/ml according to LAL assays.

#### 25 EXAMPLE 16 – CLONING AND EXPRESSION OF A POLYPEPTIDE IN A BACULOVIRUS EXPRESSION SYSTEM

In this example, the plasmid shuttle vector pAc373 is used to insert a polynucleotide into a baculovirus to express a polypeptide. A typical baculovirus expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction  
30 sites, which may include, for example BamHI, Xba I and Asp718. The polyadenylation site of the simian virus 40 ("SV40") is often used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the

beta-galactosidase gene from *E. coli* under control of a weak *Drosophila* promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate a viable virus that  
5 express the cloned polynucleotide.

Many other baculovirus vectors can be used in place of the vector above, such as pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required.  
10 Such vectors are described, for instance, in Luckow et al., *Virology* 170:31-39 (1989).

A polynucleotide encoding a polypeptide of the present invention is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' ends of the DNA sequence, as outlined in Example 13, to synthesize insertion fragments. The primers used to amplify the cDNA insert should preferably contain restriction sites at the 5'  
15 end of the primers in order to clone the amplified product into the expression vector. Specifically, the cDNA sequence contained in the deposited clone, including the AUG initiation codon and the naturally associated leader sequence identified elsewhere herein (if applicable), is amplified using the PCR protocol described in Example 13. If the naturally occurring signal sequence is used to produce the protein, the vector used  
20 does not need a second signal peptide. Alternatively, the vector can be modified to include a baculovirus leader sequence, using the standard methods described in Summers et al., "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures" Texas Agricultural Experimental Station Bulletin No. 1555 (1987).

25 The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with appropriate restriction enzymes and again purified on a 1% agarose gel.

The plasmid is digested with the corresponding restriction enzymes and  
30 optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.).



The fragment and the dephosphorylated plasmid are ligated together with T4 DNA ligase. *E. coli* HB101 or other suitable *E. coli* hosts such as XL-1 Blue (Stratagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria containing the plasmid are identified by  
5 digesting DNA from individual colonies and analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing.

Five ug of a plasmid containing the polynucleotide is co-transformed with 1.0 ug of a commercially available linearized baculovirus DNA ("BaculoGoldtm  
10 baculovirus DNA", Pharmingen, San Diego, CA), using the lipofection method described by Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7417 (1987). One ug of BaculoGoldtm virus DNA and 5ug of the plasmid are mixed in a sterile well of a microtiter plate containing 50ul of serum-free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards, 10 ul Lipofectin plus 90 ul Grace's medium are  
15 added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is then incubated for 5 hours at 27 degrees C. The transfection solution is then removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf  
20 serum is added. Cultivation is then continued at 27 degrees C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, *supra*. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of  
25 a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10.) After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 ul of Grace's medium and the  
30 suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4 degree C.

To verify the expression of the polypeptide, Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus containing the polynucleotide at a multiplicity of infection ("MOI") of about 2. If radiolabeled proteins are desired, 6 hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Rockville, MD). After 42 hours, 5 uCi of 35S-methionine and 5 uCi 35S-cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled).

Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the produced protein.

#### EXAMPLE 17 – EXPRESSION OF A POLYPEPTIDE IN MAMMALIAN CELLS

The polypeptide of the present invention can be expressed in a mammalian cell. A typical mammalian expression vector contains a promoter element, which mediates the initiation of transcription of mRNA, a protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription is achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter).

Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146), pBC12MI (ATCC 67109), pCMVSPORT 2.0, and pCMVSPORT 3.0. Mammalian host cells that could be used include, human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the polypeptide can be expressed in stable cell lines containing the polynucleotide integrated into a chromosome. The co-transformation with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transformed cells.

5           The transformed gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful in developing cell lines that carry several hundred or even several thousand copies of the gene of interest. (See, e.g., Alt, F. W., et al., J. Biol. Chem.... 253:1357-1370 (1978); Hamlin, J. L. and Ma, C., Biochem. et Biophys. Acta, 1097:107-143 (1990); Page, M. J. and  
10       Sydenham, M. A., Biotechnology 9:64-68 (1991).) Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., Biochem J. 227:277-279 (1991); Bebbington et al., Bio/Technology 10:169-175 (1992). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a  
15       chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

          A polynucleotide of the present invention is amplified according to the protocol outlined in herein. If the naturally occurring signal sequence is used to produce the protein, the vector does not need a second signal peptide. Alternatively, if  
20       the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., WO 96/34891.) The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("GeneClean" BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with appropriate restriction enzymes and again purified on a 1% agarose gel.

25           The amplified fragment is then digested with the same restriction enzyme and purified on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. E. coli HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC6 using, for instance, restriction enzyme analysis.

30           Chinese hamster ovary cells lacking an active DHFR gene is used for transformation. Five µg of an expression plasmid is cotransformed with 0.5 ug of the plasmid pSVneo using lipofectin (Felgner et al., supra). The plasmid pSV2-neo

contains a dominant selectable marker, the neo gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 uM, 2 uM, 5 uM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 uM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

EXAMPLE 18 – METHOD OF CREATING N- AND C-TERMINAL DELETION  
MUTANTS CORRESPONDING TO THE BGS-42 POLYPEPTIDE  
OF THE PRESENT INVENTION

As described elsewhere herein, the present invention encompasses the creation of N- and C-terminal deletion mutants, in addition to any combination of N- and C-terminal deletions thereof, corresponding to the BGS-42 polypeptide of the present invention. A number of methods are available to one skilled in the art for creating such mutants. Such methods may include a combination of PCR amplification and gene cloning methodology. Although one of skill in the art of molecular biology, through the use of the teachings provided or referenced herein, and/or otherwise known in the art as standard methods, could readily create each deletion mutant of the present invention, exemplary methods are described below.

Briefly, using the isolated cDNA clone encoding the full-length BGS-42 polypeptide sequence (as described in Example 13, for example), appropriate primers of about 15-25 nucleotides derived from the desired 5' and 3' positions of SEQ ID NO:1 may be designed to PCR amplify, and subsequently clone, the intended N- and/or C-terminal deletion mutant. Such primers could comprise, for example, an initiation and stop codon for the 5' and 3' primer, respectively. Such primers may

also comprise restriction sites to facilitate cloning of the deletion mutant post amplification. Moreover, the primers may comprise additional sequences, such as, for example, flag-tag sequences, kozac sequences, or other sequences discussed and/or referenced herein.

- 5 For example, in the case of the E89 to G306 N-terminal deletion mutant, the following primers could be used to amplify a cDNA fragment corresponding to this deletion mutant:

5' Primer	5'-GCAGCA <u>GCGGCCGC</u> GCCGGGAGCAGCGACCTGAGCAGC -3' (SEQ ID NO:63) NotI
3' Primer	5'- GCAGCA <u>GTCGAC</u> TGAACCTTTTCCTCCGGGCGGCGG -3' (SEQ ID NO:64) SalI

- 10 For example, in the case of the M1 to D171 C-terminal deletion mutant, the following primers could be used to amplify a cDNA fragment corresponding to this deletion mutant:

5' Primer	5'- GCAGCA <u>GCGGCCGC</u> ATGGCATCCAGCATCCTCAAGTGGG -3' (SEQ ID NO:65) NotI
3' Primer	5'- GCAGCA <u>GTCGAC</u> GCCGATGTACAGCTGCGGTCCACG -3' (SEQ ID NO:66) SalI

- 15 Representative PCR amplification conditions are provided below, although the skilled artisan would appreciate that other conditions may be required for efficient amplification. A 100 ul PCR reaction mixture may be prepared using 10ng of the template DNA (cDNA clone of BGS-42), 200 uM 4dNTPs, 1uM primers, 0.25U Taq DNA polymerase (PE), and standard Taq DNA polymerase buffer. Typical PCR  
20 cycling condition are as follows:

- 20-25 cycles: 45 sec, 93 degrees  
2 min, 50 degrees  
2 min, 72 degrees  
25 1 cycle: 10 min, 72 degrees

After the final extension step of PCR, 5U Klenow Fragment may be added and incubated for 15 min at 30 degrees.

Upon digestion of the fragment with the NotI and SalI restriction enzymes, the fragment could be cloned into an appropriate expression and/or cloning vector which has been similarly digested (e.g., pSport1, among others). . The skilled artisan would appreciate that other plasmids could be equally substituted, and may be desirable in certain circumstances. The digested fragment and vector are then ligated using a DNA ligase, and then used to transform competent E.coli cells using methods provided herein and/or otherwise known in the art.

The 5' primer sequence for amplifying any additional N-terminal deletion mutants may be determined by reference to the following formula:  $(S+(X * 3))$  to  $((S+(X * 3))+25)$ , wherein 'S' is equal to the nucleotide position of the initiating start codon of the BGS-42 gene (SEQ ID NO:1), and 'X' is equal to the most N-terminal amino acid of the intended N-terminal deletion mutant. The first term will provide the start 5' nucleotide position of the 5' primer, while the second term will provide the end 3' nucleotide position of the 5' primer corresponding to sense strand of SEQ ID NO:1. Once the corresponding nucleotide positions of the primer are determined, the final nucleotide sequence may be created by the addition of applicable restriction site sequences to the 5' end of the sequence, for example. As referenced herein, the addition of other sequences to the 5' primer may be desired in certain circumstances (e.g., kozac sequences, etc.).

The 3' primer sequence for amplifying any additional N-terminal deletion mutants may be determined by reference to the following formula:  $(S+(X * 3))$  to  $((S+(X * 3))-25)$ , wherein 'S' is equal to the nucleotide position of the initiating start codon of the BGS-42 gene (SEQ ID NO:1), and 'X' is equal to the most C-terminal amino acid of the intended N-terminal deletion mutant. The first term will provide the start 5' nucleotide position of the 3' primer, while the second term will provide the end 3' nucleotide position of the 3' primer corresponding to the anti-sense strand of SEQ ID NO:1. Once the corresponding nucleotide positions of the primer are determined, the final nucleotide sequence may be created by the addition of applicable restriction site sequences to the 5' end of the sequence, for example. As referenced herein, the addition of other sequences to the 3' primer may be desired in certain circumstances (e.g., stop codon sequences, etc.). The skilled artisan would

appreciate that modifications of the above nucleotide positions may be necessary for optimizing PCR amplification.

The same general formulas provided above may be used in identifying the 5' and 3' primer sequences for amplifying any C-terminal deletion mutant of the present invention. Moreover, the same general formulas provided above may be used in identifying the 5' and 3' primer sequences for amplifying any combination of N-terminal and C-terminal deletion mutant of the present invention. The skilled artisan would appreciate that modifications of the above nucleotide positions may be necessary for optimizing PCR amplification.

#### EXAMPLE 19 – PROTEIN FUSIONS

The polypeptides of the present invention are preferably fused to other proteins. These fusion proteins can be used for a variety of applications. For example, fusion of the present polypeptides to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See Example described herein; see also EP A 394,827; Traunecker, et al., Nature 331:84-86 (1988).) Similarly, fusion to IgG-1, IgG-3, and albumin increases the half-life time in vivo. Nuclear localization signals fused to the polypeptides of the present invention can target the protein to a specific subcellular localization, while covalent heterodimer or homodimers can increase or decrease the activity of a fusion protein. Fusion proteins can also create chimeric molecules having more than one function. Finally, fusion proteins can increase solubility and/or stability of the fused protein compared to the non-fused protein. All of the types of fusion proteins described above can be made by modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule.

Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described below. These primers also should have convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian expression vector. Note that the polynucleotide is cloned without a stop codon, otherwise a fusion protein will not be produced.

The naturally occurring signal sequence may be used to produce the protein (if applicable). Alternatively, if the naturally occurring signal sequence is not used, the

vector can be modified to include a heterologous signal sequence. (See, e.g., WO 96/34891 and/or US Patent No. 6,066,781, *supra*.)

Human IgG Fc region:

5 GGGATCCGGAGCCCAAATCTTCTGACAAAACTCACACATGCCCACCGTGC  
 CCAGCACCTGAATTCGAGGGTGCACCGTCAGTCTTCCTCTTCCCCCAAAA  
 CCCAAGGACACCCTCATGATCTCCCGGACTCCTGAGGTCACATGCGTGGT  
 GGTGGACGTAAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGG  
 ACGGCGTGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTA  
 10 CAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACT  
 GGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCA  
 ACCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAAC  
 CACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAG  
 GTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAAGCGACATCGCCGT  
 15 GGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACACTACAAGACCACGCCT  
 CCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTG  
 GACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCA  
 TGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGG  
 GTAAATGAGTGCGACGGCCGCGACTCTAGAGGAT (SEQ ID NO:62)

20

EXAMPLE 20 – REGULATION OF PROTEIN EXPRESSION VIA  
 CONTROLLED AGGREGATION IN THE ENDOPLASMIC RETICULUM

As described more particularly herein, proteins regulate diverse cellular  
 processes in higher organisms, ranging from rapid metabolic changes to growth and  
 25 differentiation. Increased production of specific proteins could be used to prevent  
 certain diseases and/or disease states. Thus, the ability to modulate the expression of  
 specific proteins in an organism would provide significant benefits.

Numerous methods have been developed to date for introducing foreign genes,  
 either under the control of an inducible, constitutively active, or endogenous  
 30 promoter, into organisms. Of particular interest are the inducible promoters (see, M.  
 Gossen, et al., *Proc. Natl. Acad. Sci. USA*, 89:5547 (1992); Y. Wang, et al., *Proc.*  
*Natl. Acad. Sci. USA*, 91:8180 (1994), D. No., et al., *Proc. Natl. Acad. Sci. USA*,



93:3346 (1996); and V.M. Rivera, et al., *Nature Med*, 2:1028 (1996); in addition to additional examples disclosed elsewhere herein). In one example, the gene for erythropoietin (Epo) was transferred into mice and primates under the control of a small molecule inducer for expression (e.g., tetracycline or rapamycin) (see, D. Bohl, et al., *Blood*, 92:1512, (1998); K.G. Rendahl, et al., *Nat. Biotech*, 16:757, (1998); V.M. Rivera, et al., *Proc. Natl. Acad. Sci. USA*, 96:8657 (1999); and X.Ye et al., *Science*, 283:88 (1999). Although such systems enable efficient induction of the gene of interest in the organism upon addition of the inducing agent (i.e., tetracycline, rapamycin, etc.), the levels of expression tend to peak at 24 hours and trail off to background levels after 4 to 14 days. Thus, controlled transient expression is virtually impossible using these systems, though such control would be desirable.

A new alternative method of controlling gene expression levels of a protein from a transgene (i.e., includes stable and transient transformants) has recently been elucidated (V.M. Rivera, et al., *Science*, 287:826-830, (2000)). This method does not control gene expression at the level of the mRNA like the aforementioned systems. Rather, the system controls the level of protein in an active secreted form. In the absence of the inducing agent, the protein aggregates in the ER and is not secreted. However, addition of the inducing agent results in dis-aggregation of the protein and the subsequent secretion from the ER. Such a system affords low basal secretion, rapid, high level secretion in the presence of the inducing agent, and rapid cessation of secretion upon removal of the inducing agent. In fact, protein secretion reached a maximum level within 30 minutes of induction, and a rapid cessation of secretion within 1 hour of removing the inducing agent. The method is also applicable for controlling the level of production for membrane proteins.

Detailed methods are presented in V.M. Rivera, et al., *Science*, 287:826-830, (2000)), briefly:

Fusion protein constructs are created using polynucleotide sequences of the present invention with one or more copies (preferably at least 2, 3, 4, or more) of a conditional aggregation domain (CAD) a domain that interacts with itself in a ligand-reversible manner (i.e., in the presence of an inducing agent) using molecular biology methods known in the art and discussed elsewhere herein. The CAD domain may be the mutant domain isolated from the human FKBP12 (Phe<sup>36</sup> to Met) protein (as

disclosed in V.M. Rivera., et al., Science, 287:826-830, (2000), or alternatively other proteins having domains with similar ligand-reversible, self-aggregation properties. As a principle of design the fusion protein vector would contain a furin cleavage sequence operably linked between the polynucleotides of the present invention and the CAD domains. Such a cleavage site would enable the proteolytic cleavage of the CAD domains from the polypeptide of the present invention subsequent to secretion from the ER and upon entry into the trans-Golgi (J.B. Denault, et al., FEBS Lett., 379:113, (1996)). Alternatively, the skilled artisan would recognize that any proteolytic cleavage sequence could be substituted for the furin sequence provided the substituted sequence is cleavable either endogenously (e.g., the furin sequence) or exogenously (e.g., post secretion, post purification, post production, etc.). The preferred sequence of each feature of the fusion protein construct, from the 5' to 3' direction with each feature being operably linked to the other, would be a promoter, signal sequence, "X" number of (CAD)<sub>x</sub> domains, the furin sequence (or other proteolytic sequence), and the coding sequence of the polypeptide of the present invention. The artisan would appreciate that the promotor and signal sequence, independent from the other, could be either the endogenous promotor or signal sequence of a polypeptide of the present invention, or alternatively, could be a heterologous signal sequence and promotor.

The specific methods described herein for controlling protein secretion levels through controlled ER aggregation are not meant to be limiting and would be generally applicable to any of the polynucleotides and polypeptides of the present invention, including variants, homologues, orthologs, and fragments therein.

#### EXAMPLE 21 – ALTERATION OF PROTEIN GLYCOSYLATION SITES TO ENHANCE CHARACTERISTICS OF POLYPEPTIDES OF THE INVENTION

Many eukaryotic cell surface and proteins are post-translationally processed to incorporate N-linked and O-linked carbohydrates (Kornfeld and Kornfeld (1985) Annu. Rev. Biochem. 54:631-64; Rademacher et al., (1988) Annu. Rev. Biochem. 57:785-838). Protein glycosylation is thought to serve a variety of functions including: augmentation of protein folding, inhibition of protein aggregation, regulation of intracellular trafficking to organelles, increasing resistance to

proteolysis, modulation of protein antigenicity, and mediation of intercellular adhesion (Fieldler and Simons (1995) *Cell*, 81:309-312; Helenius (1994) *Mol. Biol. Of the Cell* 5:253-265; Olden et al., (1978) *Cell*, 13:461-473; Caton et al., (1982) *Cell*, 37:417-427; Alexamnder and Elder (1984), *Science*, 226:1328-1330; and Flack et al.,  
 5 (1994), *J. Biol. Chem.*, 269:14015-14020). In higher organisms, the nature and extent of glycosylation can markedly affect the circulating half-life and bio-availability of proteins by mechanisms involving receptor mediated uptake and clearance (Ashwell and Morrell, (1974), *Adv. Enzymol.*, 41:99-128; Ashwell and Harford (1982), *Ann. Rev. Biochem.*, 51:531-54). Receptor systems have been identified that are thought to  
 10 play a major role in the clearance of serum proteins through recognition of various carbohydrate structures on the glycoproteins (Stockert (1995), *Physiol. Rev.*, 75:591-609; Kery et al., (1992), *Arch. Biochem. Biophys.*, 298:49-55). Thus, production strategies resulting in incomplete attachment of terminal sialic acid residues might provide a means of shortening the bioavailability and half-life of glycoproteins.  
 15 Conversely, expression strategies resulting in saturation of terminal sialic acid attachment sites might lengthen protein bioavailability and half-life.

In the development of recombinant glycoproteins for use as pharmaceutical products, for example, it has been speculated that the pharmacodynamics of recombinant proteins can be modulated by the addition or deletion of glycosylation  
 20 sites from a glycoproteins primary structure (Berman and Lasky (1985a) *Trends in Biotechnol.*, 3:51-53). However, studies have reported that the deletion of N-linked glycosylation sites often impairs intracellular transport and results in the intracellular accumulation of glycosylation site variants (Machamer and Rose (1988), *J. Biol Chem.*, 263:5955-5960; Gallagher et al., (1992), *J. Virology.*, 66:7136-7145; Collier  
 25 et al., (1993), *Biochem.*, 32:7818-7823; Claffey et al., (1995) *Biochemica et Biophysica Acta*, 1246:1-9; Dube et al., (1988), *J. Biol. Chem.* 263:17516-17521). While glycosylation site variants of proteins can be expressed intracellularly, it has proved difficult to recover useful quantities from growth conditioned cell culture medium.

30 Moreover, it is unclear to what extent a glycosylation site in one species will be recognized by another species glycosylation machinery. Due to the importance of glycosylation in protein metabolism, particularly the secretion and/or expression of

the protein, whether a glycosylation signal is recognized may profoundly determine a proteins ability to be expressed, either endogenously or recombinately, in another organism (i.e., expressing a human protein in E.coli, yeast, or viral organisms; or an E.coli, yeast, or viral protein in human, etc.). Thus, it may be desirable to add, delete,  
5 or modify a glycosylation site, and possibly add a glycosylation site of one species to a protein of another species to improve the proteins functional, bioprocess purification, and/or structural characteristics (e.g., a polypeptide of the present invention).

A number of methods may be employed to identify the location of  
10 glycosylation sites within a protein. One preferred method is to run the translated protein sequence through the PROSITE computer program (Swiss Institute of Bioinformatics). Once identified, the sites could be systematically deleted, or impaired, at the level of the DNA using mutagenesis methodology known in the art and available to the skilled artisan, Preferably using PCR-directed mutagenesis (See  
15 Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring, NY (1982)). Similarly, glycosylation sites could be added, or modified at the level of the DNA using similar methods, preferably PCR methods (See, Maniatis, supra). The results of modifying the glycosylation sites for a particular protein (e.g., solubility, secretion potential, activity, aggregation, proteolytic resistance, etc.) could  
20 then be analyzed using methods know in the art.

The skilled artisan would acknowledge the existence of other computer algorithms capable of predicting the location of glycosylation sites within a protein. For example, the Motif computer program (Genetics Computer Group suite of  
25 programs) provides this function, as well.

#### EXAMPLE 22 – METHOD OF ENHANCING THE BIOLOGICAL ACTIVITY/FUNCTIONAL CHARACTERISTICS OF INVENTION THROUGH MOLECULAR EVOLUTION

Although many of the most biologically active proteins known are highly  
30 effective for their specified function in an organism, they often possess characteristics that make them undesirable for transgenic, therapeutic, and/or industrial applications. Among these traits, a short physiological half-life is the most prominent problem, and

is present either at the level of the protein, or the level of the proteins mRNA. The ability to extend the half-life, for example, would be particularly important for a proteins use in gene therapy, transgenic animal production, the bioprocess production and purification of the protein, and use of the protein as a chemical modulator among  
5 others. Therefore, there is a need to identify novel variants of isolated proteins possessing characteristics which enhance their application as a therapeutic for treating diseases of animal origin, in addition to the proteins applicability to common industrial and pharmaceutical applications.

Thus, one aspect of the present invention relates to the ability to enhance  
10 specific characteristics of invention through directed molecular evolution. Such an enhancement may, in a non-limiting example, benefit the inventions utility as an essential component in a kit, the inventions physical attributes such as its solubility, structure, or codon optimization, the inventions specific biological activity, including any associated enzymatic activity, the proteins enzyme kinetics, the proteins  $K_i$ ,  $K_{cat}$ ,  
15  $K_m$ ,  $V_{max}$ ,  $K_d$ , protein-protein activity, protein-DNA binding activity, antagonist/inhibitory activity (including direct or indirect interaction), agonist activity (including direct or indirect interaction), the proteins antigenicity (e.g., where it would be desirable to either increase or decrease the antigenic potential of the protein), the immunogenicity of the protein, the ability of the protein to form dimers, trimers, or  
20 multimers with either itself or other proteins, the antigenic efficacy of the invention, including its subsequent use a preventative treatment for disease or disease states, or as an effector for targeting diseased genes. Moreover, the ability to enhance specific characteristics of a protein may also be applicable to changing the characterized activity of an enzyme to an activity completely unrelated to its initially characterized  
25 activity. Other desirable enhancements of the invention would be specific to each individual protein, and would thus be well known in the art and contemplated by the present invention.

For example, an engineered tubulin tyrosine ligase protein may have altered specificity for its cognate receptor. In yet another example, an engineered tubulin  
30 tyrosine ligase protein may be capable of being activated with less than all of the regulatory factors and/or conditions typically required for tubulin tyrosine ligase protein activation (e.g., phosphorylation, conformational changes, etc.). Such a

tubulin tyrosine ligase protein would be useful in screens to identify tubulin tyrosine ligase protein modulators, among other uses described herein.

Directed evolution is comprised of several steps. The first step is to establish a library of variants for the gene or protein of interest. The most important step is to then select for those variants that entail the activity you wish to identify. The design of the screen is essential since your screen should be selective enough to eliminate non-useful variants, but not so stringent as to eliminate all variants. The last step is then to repeat the above steps using the best variant from the previous screen. Each successive cycle, can then be tailored as necessary, such as increasing the stringency of the screen, for example.

Over the years, there have been a number of methods developed to introduce mutations into macromolecules. Some of these methods include, random mutagenesis, “error-prone” PCR, chemical mutagenesis, site-directed mutagenesis, and other methods well known in the art (for a comprehensive listing of current mutagenesis methods, see Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring, NY (1982)). Typically, such methods have been used, for example, as tools for identifying the core functional region(s) of a protein or the function of specific domains of a protein (if a multi-domain protein). However, such methods have more recently been applied to the identification of macromolecule variants with specific or enhanced characteristics.

Random mutagenesis has been the most widely recognized method to date. Typically, this has been carried out either through the use of “error-prone” PCR (as described in Moore, J., et al, *Nature Biotechnology* 14:458, (1996), or through the application of randomized synthetic oligonucleotides corresponding to specific regions of interest (as described by Derbyshire, K.M. et al, *Gene*, 46:145-152, (1986), and Hill, DE, et al, *Methods Enzymol.*, 55:559-568, (1987). Both approaches have limits to the level of mutagenesis that can be obtained. However, either approach enables the investigator to effectively control the rate of mutagenesis. This is particularly important considering the fact that mutations beneficial to the activity of the enzyme are fairly rare. In fact, using too high a level of mutagenesis may counter or inhibit the desired benefit of a useful mutation.

While both of the aforementioned methods are effective for creating randomized pools of macromolecule variants, a third method, termed "DNA Shuffling", or "sexual PCR" (WPC, Stemmer, PNAS, 91:10747, (1994)) has recently been elucidated. DNA shuffling has also been referred to as "directed molecular evolution", "exon-shuffling", "directed enzyme evolution", "in vitro evolution", and "artificial evolution". Such reference terms are known in the art and are encompassed by the invention. This new, preferred, method apparently overcomes the limitations of the previous methods in that it not only propagates positive traits, but simultaneously eliminates negative traits in the resulting progeny.

DNA shuffling accomplishes this task by combining the principal of in vitro recombination, along with the method of "error-prone" PCR. In effect, you begin with a randomly digested pool of small fragments of your gene, created by Dnase I digestion, and then introduce said random fragments into an "error-prone" PCR assembly reaction. During the PCR reaction, the randomly sized DNA fragments not only hybridize to their cognate strand, but also may hybridize to other DNA fragments corresponding to different regions of the polynucleotide of interest – regions not typically accessible via hybridization of the entire polynucleotide. Moreover, since the PCR assembly reaction utilizes "error-prone" PCR reaction conditions, random mutations are introduced during the DNA synthesis step of the PCR reaction for all of the fragments -further diversifying the potential hybridization sites during the annealing step of the reaction.

A variety of reaction conditions could be utilized to carry-out the DNA shuffling reaction. However, specific reaction conditions for DNA shuffling are provided, for example, in PNAS, 91:10747, (1994). Briefly:

Prepare the DNA substrate to be subjected to the DNA shuffling reaction. Preparation may be in the form of simply purifying the DNA from contaminating cellular material, chemicals, buffers, oligonucleotide primers, deoxynucleotides, RNAs, etc., and may entail the use of DNA purification kits as those provided by Qiagen, Inc., or by the Promega, Corp., for example.

Once the DNA substrate has been purified, it would be subjected to Dnase I digestion. About 2-4ug of the DNA substrate(s) would be digested with .0015 units of Dnase I (Sigma) per ul in 100ul of 50mM Tris-HCL, pH 7.4/1mM MgCl<sub>2</sub> for 10-20

min. at room temperature. The resulting fragments of 10-50bp could then be purified by running them through a 2% low-melting point agarose gel by electrophoresis onto DE81 ion-exchange paper (Whatmann) or could be purified using Microcon concentrators (Amicon) of the appropriate molecular weight cutoff, or could use  
5 oligonucleotide purification columns (Qiagen), in addition to other methods known in the art. If using DE81 ion-exchange paper, the 10-50bp fragments could be eluted from said paper using 1M NaCl, followed by ethanol precipitation.

The resulting purified fragments would then be subjected to a PCR assembly reaction by re-suspension in a PCR mixture containing: 2mM of each dNTP, 2.2mM  
10 MgCl<sub>2</sub>, 50 mM KCl, 10mM Tris•HCL, pH 9.0, and 0.1% Triton X-100, at a final fragment concentration of 10-30ng/ul. No primers are added at this point. *Taq* DNA polymerase (Promega) would be used at 2.5 units per 100ul of reaction mixture. A PCR program of 94 C for 60s; 94 C for 30s, 50-55 C for 30s, and 72 C for 30s using 30-45 cycles, followed by 72 C for 5min using an MJ Research (Cambridge, MA)  
15 PTC-150 thermocycler. After the assembly reaction is completed, a 1:40 dilution of the resulting primerless product would then be introduced into a PCR mixture (using the same buffer mixture used for the assembly reaction) containing 0.8um of each primer and subjecting this mixture to 15 cycles of PCR (using 94 C for 30s, 50 C for 30s, and 72 C for 30s). The referred primers would be primers corresponding to the  
20 nucleic acid sequences of the polynucleotide(s) utilized in the shuffling reaction. Said primers could consist of modified nucleic acid base pairs using methods known in the art and referred to else where herein, or could contain additional sequences (i.e., for adding restriction sites, mutating specific base-pairs, etc.).

The resulting shuffled, assembled, and amplified product can be purified using  
25 methods well known in the art (e.g., Qiagen PCR purification kits) and then subsequently cloned using appropriate restriction enzymes.

Although a number of variations of DNA shuffling have been published to date, such variations would be obvious to the skilled artisan and are encompassed by the invention. The DNA shuffling method can also be tailored to the desired level of  
30 mutagenesis using the methods described by Zhao, et al. (Nucl Acid Res., 25(6):1307-1308, (1997)).



As described above, once the randomized pool has been created, it can then be subjected to a specific screen to identify the variant possessing the desired characteristic(s). Once the variant has been identified, DNA corresponding to the variant could then be used as the DNA substrate for initiating another round of DNA shuffling. This cycle of shuffling, selecting the optimized variant of interest, and then re-shuffling, can be repeated until the ultimate variant is obtained. Examples of model screens applied to identify variants created using DNA shuffling technology may be found in the following publications: J. C., Moore, et al., *J. Mol. Biol.*, 272:336-347, (1997), F.R., Cross, et al., *Mol. Cell. Biol.*, 18:2923-2931, (1998), and A. Cramer, et al., *Nat. Biotech.*, 15:436-438, (1997).

DNA shuffling has several advantages. First, it makes use of beneficial mutations. When combined with screening, DNA shuffling allows the discovery of the best mutational combinations and does not assume that the best combination contains all the mutations in a population. Secondly, recombination occurs simultaneously with point mutagenesis. An effect of forcing DNA polymerase to synthesize full-length genes from the small fragment DNA pool is a background mutagenesis rate. In combination with a stringent selection method, enzymatic activity has been evolved up to 16000 fold increase over the wild-type form of the enzyme. In essence, the background mutagenesis yielded the genetic variability on which recombination acted to enhance the activity.

A third feature of recombination is that it can be used to remove deleterious mutations. As discussed above, during the process of the randomization, for every one beneficial mutation, there may be at least one or more neutral or inhibitory mutations. Such mutations can be removed by including in the assembly reaction an excess of the wild-type random-size fragments, in addition to the random-size fragments of the selected mutant from the previous selection. During the next selection, some of the most active variants of the polynucleotide/polypeptide/enzyme, should have lost the inhibitory mutations.

Finally, recombination enables parallel processing. This represents a significant advantage since there are likely multiple characteristics that would make a protein more desirable (e.g. solubility, activity, etc.). Since it is increasingly difficult to screen for more than one desirable trait at a time, other methods of molecular

evolution tend to be inhibitory. However, using recombination, it would be possible to combine the randomized fragments of the best representative variants for the various traits, and then select for multiple properties at once.

DNA shuffling can also be applied to the polynucleotides and polypeptides of the present invention to decrease their immunogenicity in a specified host. For example, a particular variant of the present invention may be created and isolated using DNA shuffling technology. Such a variant may have all of the desired characteristics, though may be highly immunogenic in a host due to its novel intrinsic structure. Specifically, the desired characteristic may cause the polypeptide to have a non-native structure which could no longer be recognized as a "self" molecule, but rather as a "foreign", and thus activate a host immune response directed against the novel variant. Such a limitation can be overcome, for example, by including a copy of the gene sequence for a xenobiotic ortholog of the native protein in with the gene sequence of the novel variant gene in one or more cycles of DNA shuffling. The molar ratio of the ortholog and novel variant DNAs could be varied accordingly. Ideally, the resulting hybrid variant identified would contain at least some of the coding sequence which enabled the xenobiotic protein to evade the host immune system, and additionally, the coding sequence of the original novel variant that provided the desired characteristics.

Likewise, the invention encompasses the application of DNA shuffling technology to the evolution of polynucleotides and polypeptides of the invention, wherein one or more cycles of DNA shuffling include, in addition to the gene template DNA, oligonucleotides coding for known allelic sequences, optimized codon sequences, known variant sequences, known polynucleotide polymorphism sequences, known ortholog sequences, known homologue sequences, additional homologous sequences, additional non-homologous sequences, sequences from another species, and any number and combination of the above.

In addition to the described methods above, there are a number of related methods that may also be applicable, or desirable in certain cases. Representative among these are the methods discussed in PCT applications WO 98/31700, and WO 98/32845, which are hereby incorporated by reference. Furthermore, related methods can also be applied to the polynucleotide sequences of the present invention in order

to evolve invention for creating ideal variants for use in gene therapy, protein engineering, evolution of whole cells containing the variant, or in the evolution of entire enzyme pathways containing polynucleotides of the invention as described in PCT applications WO 98/13485, WO 98/13487, WO 98/27230, WO 98/31837, and  
5 Cramer, A., et al., Nat. Biotech., 15:436-438, (1997), respectively.

Additional methods of applying "DNA Shuffling" technology to the polynucleotides and polypeptides of the present invention, including their proposed applications, may be found in US Patent No. 5,605,793; PCT Application No. WO 95/22625; PCT Application No. WO 97/20078; PCT Application No. WO 97/35966;  
10 and PCT Application No. WO 98/42832; PCT Application No. WO 00/09727 specifically provides methods for applying DNA shuffling to the identification of herbicide selective crops which could be applied to the polynucleotides and polypeptides of the present invention; additionally, PCT Application No. WO 00/12680 provides methods and compositions for generating, modifying, adapting,  
15 and optimizing polynucleotide sequences that confer detectable phenotypic properties on plant species; each of the above are hereby incorporated in their entirety herein for all purposes.

#### 20 EXAMPLE 23 – METHOD OF DETERMINING ALTERATIONS IN A GENE CORRESPONDING TO A POLYNUCLEOTIDE

RNA isolated from entire families or individual patients presenting with a phenotype of interest (such as a disease) is be isolated. cDNA is then generated from these RNA samples using protocols known in the art. (See, Sambrook.) The cDNA is then used as a template for PCR, employing primers surrounding regions of interest in  
25 SEQ ID NO:1. Suggested PCR conditions consist of 35 cycles at 95 degrees C for 30 seconds; 60-120 seconds at 52-58 degrees C; and 60-120 seconds at 70 degrees C, using buffer solutions described in Sidransky et al., Science 252:706 (1991).

PCR products are then sequenced using primers labeled at their 5' end with T4 polynucleotide kinase, employing SequiTherm Polymerase. (Epicentre Technologies).  
30 The intron-exon borders of selected exons is also determined and genomic PCR products analyzed to confirm the results. PCR products harboring suspected mutations is then cloned and sequenced to validate the results of the direct sequencing.

PCR products are cloned into T-tailed vectors as described in Holton et al., Nucleic Acids Research, 19:1156 (1991) and sequenced with T7 polymerase (United States Biochemical). Affected individuals are identified by mutations not present in unaffected individuals.

5        Genomic rearrangements are also observed as a method of determining alterations in a gene corresponding to a polynucleotide. Genomic clones isolated according to the methods described herein are nick-translated with digoxigenindeoxyuridine 5'-triphosphate (Boehringer Mannheim), and FISH performed as described in Johnson et al., Methods Cell Biol. 35:73-99 (1991). Hybridization with the labeled  
10       probe is carried out using a vast excess of human cot-1 DNA for specific hybridization to the corresponding genomic locus.

Chromosomes are counterstained with 4,6-diamino-2-phenylidole and propidium iodide, producing a combination of C- and R-bands. Aligned images for precise mapping are obtained using a triple-band filter set (Chroma Technology,  
15       Brattleboro, VT) in combination with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and variable excitation wavelength filters. (Johnson et al., Genet. Anal. Tech. Appl., 8:75 (1991).) Image collection, analysis and chromosomal fractional length measurements are performed using the ISee Graphical Program System. (Inovision Corporation, Durham, NC.) Chromosome alterations of the  
20       genomic region hybridized by the probe are identified as insertions, deletions, and translocations. These alterations are used as a diagnostic marker for an associated disease.

#### 25        EXAMPLE 24 – METHOD OF DETECTING ABNORMAL LEVELS OF A POLYPEPTIDE IN A BIOLOGICAL SAMPLE

A polypeptide of the present invention can be detected in a biological sample, and if an increased or decreased level of the polypeptide is detected, this polypeptide is a marker for a particular phenotype. Methods of detection are numerous, and thus, it is understood that one skilled in the art can modify the following assay to fit their  
30       particular needs.

For example, antibody-sandwich ELISAs are used to detect polypeptides in a sample, preferably a biological sample. Wells of a microtiter plate are coated with

specific antibodies, at a final concentration of 0.2 to 10 ug/ml. The antibodies are either monoclonal or polyclonal and are produced by the method described elsewhere herein. The wells are blocked so that non-specific binding of the polypeptide to the well is reduced.

5           The coated wells are then incubated for > 2 hours at RT with a sample containing the polypeptide. Preferably, serial dilutions of the sample should be used to validate results. The plates are then washed three times with deionized or distilled water to remove unbounded polypeptide.

          Next, 50 ul of specific antibody-alkaline phosphatase conjugate, at a  
10       concentration of 25-400 ng, is added and incubated for 2 hours at room temperature. The plates are again washed three times with deionized or distilled water to remove unbounded conjugate..

          Add 75 ul of 4-methylumbelliferyl phosphate (MUP) or p-nitrophenyl phosphate (NPP) substrate solution to each well and incubate 1 hour at room  
15       temperature. Measure the reaction by a microtiter plate reader. Prepare a standard curve, using serial dilutions of a control sample, and plot polypeptide concentration on the X-axis (log scale) and fluorescence or absorbance of the Y-axis (linear scale). Interpolate the concentration of the polypeptide in the sample using the standard curve.

20

#### EXAMPLE 25 – FORMULATION

          The invention also provides methods of treatment and/or prevention diseases, disorders, and/or conditions (such as, for example, any one or more of the diseases or disorders disclosed herein) by administration to a subject of an effective amount of a  
25       Therapeutic. By therapeutic is meant a polynucleotides or polypeptides of the invention (including fragments and variants), agonists or antagonists thereof, and/or antibodies thereto, in combination with a pharmaceutically acceptable carrier type (e.g., a sterile carrier).

          The Therapeutic will be formulated and dosed in a fashion consistent with  
30       good medical practice, taking into account the clinical condition of the individual patient (especially the side effects of treatment with the Therapeutic alone), the site of delivery, the method of administration, the scheduling of administration, and other

factors known to practitioners. The "effective amount" for purposes herein is thus determined by such considerations.

As a general proposition, the total pharmaceutically effective amount of the Therapeutic administered parenterally per dose will be in the range of about 1 ug/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the Therapeutic is typically administered at a dose rate of about 1 ug/kg/hour to about 50 ug/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The length of treatment needed to observe changes and the interval following treatment for responses to occur appears to vary depending on the desired effect.

Therapeutics can be administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), buccally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

In yet an additional embodiment, the Therapeutics of the invention are delivered orally using the drug delivery technology described in U.S. Patent 6,258,789, which is hereby incorporated by reference herein.

Therapeutics of the invention are also suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics are administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), buccally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Therapeutics of the invention may also be suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics include suitable polymeric materials (such as, for example, semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules), suitable hydrophobic materials  
5 (for example as an emulsion in an acceptable oil) or ion exchange resins, and sparingly soluble derivatives (such as, for example, a sparingly soluble salt).

Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman et al., Biopolymers 22:547-556 (1983)), poly (2- hydroxyethyl methacrylate) (Langer et al.,  
10 J. Biomed. Mater. Res. 15:167-277 (1981), and Langer, Chem. Tech. 12:98-105 (1982)), ethylene vinyl acetate (Langer et al., Id.) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988).

Sustained-release Therapeutics also include liposomally entrapped Therapeutics of the invention (see, generally, Langer, Science 249:1527-1533 (1990);  
15 Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 317 -327 and 353-365 (1989)). Liposomes containing the Therapeutic are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. (USA) 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci.(USA) 77:4030-4034 (1980); EP 52,322; EP 36,676; EP  
20 88,046; EP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal Therapeutic.

25 In yet an additional embodiment, the Therapeutics of the invention are delivered by way of a pump (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)).

Other controlled release systems are discussed in the review by Langer  
30 (Science 249:1527-1533 (1990)).

For parenteral administration, in one embodiment, the Therapeutic is formulated generally by mixing it at the desired degree of purity, in a unit dosage

injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to the Therapeutic.

Generally, the formulations are prepared by contacting the Therapeutic uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.

The Therapeutic will typically be formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be understood that the use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of polypeptide salts.

Any pharmaceutical used for therapeutic administration can be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutics generally are placed into a container having a



sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Therapeutics ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized  
5 formulation for reconstitution. As an example of a lyophilized formulation, 10-ml vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous Therapeutic solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized Therapeutic using bacteriostatic Water-for-Injection.

The invention also provides a pharmaceutical pack or kit comprising one or  
10 more containers filled with one or more of the ingredients of the Therapeutics of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the Therapeutics may be employed in  
15 conjunction with other therapeutic compounds.

The Therapeutics of the invention may be administered alone or in combination with adjuvants. Adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, alum, alum plus deoxycholate (ImmunoAg), MTP-PE (Biocine Corp.), QS21 (Genentech, Inc.), BCG,  
20 and MPL. In a specific embodiment, Therapeutics of the invention are administered in combination with alum. In another specific embodiment, Therapeutics of the invention are administered in combination with QS-21. Further adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, Monophosphoryl lipid immunomodulator, AdjuVax 100a, QS-21, QS-18, CRL1005,  
25 Aluminum salts, MF-59, and Virosomal adjuvant technology. Vaccines that may be administered with the Therapeutics of the invention include, but are not limited to, vaccines directed toward protection against MMR (measles, mumps, rubella), polio, varicella, tetanus/diphtheria, hepatitis A, hepatitis B, haemophilus influenzae B, whooping cough, pneumonia, influenza, Lyme's Disease, rotavirus, cholera, yellow  
30 fever, Japanese encephalitis, poliomyelitis, rabies, typhoid fever, and pertussis. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes

presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate  
5 administration of one of the compounds or agents given first, followed by the second.

The Therapeutics of the invention may be administered alone or in combination with other therapeutic agents. Therapeutic agents that may be administered in combination with the Therapeutics of the invention, include but not limited to, other members of the TNF family, chemotherapeutic agents, antibiotics,  
10 steroidal and non-steroidal anti-inflammatories, conventional immunotherapeutic agents, cytokines and/or tubulin tyrosine ligase proteins. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also  
15 procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

In one embodiment, the Therapeutics of the invention are administered in  
20 combination with members of the TNF family. TNF, TNF-related or TNF-like molecules that may be administered with the Therapeutics of the invention include, but are not limited to, soluble forms of TNF-alpha, lymphotoxin-alpha (LT-alpha, also known as TNF-beta), LT-beta (found in complex heterotrimer LT-alpha2-beta), OPGL, FasL, CD27L, CD30L, CD40L, 4-1BBL, DcR3, OX40L, TNF-gamma  
25 (International Publication No. WO 96/14328), AIM-I (International Publication No. WO 97/33899), endokine-alpha (International Publication No. WO 98/07880), TR6 (International Publication No. WO 98/30694), OPG, and neutrokin-alpha (International Publication No. WO 98/18921, OX40, and nerve tubulin tyrosine ligase protein (NGF), and soluble forms of Fas, CD30, CD27, CD40 and 4-IBB, TR2  
30 (International Publication No. WO 96/34095), DR3 (International Publication No. WO 97/33904), DR4 (International Publication No. WO 98/32856), TR5 (International Publication No. WO 98/30693), TR6 (International Publication No.

WO 98/30694), TR7 (International Publication No. WO 98/41629), TRANK, TR9 (International Publication No. WO 98/56892), TR10 (International Publication No. WO 98/54202), 312C2 (International Publication No. WO 98/06842), and TR12, and soluble forms CD154, CD70, and CD153.

5           In certain embodiments, Therapeutics of the invention are administered in combination with antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors. Nucleoside reverse transcriptase inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, RETROVIR  
10   (zidovudine/AZT), VIDEX (didanosine/ddI), HIVID (zalcitabine/ddC), ZERIT (stavudine/d4T), EPIVIR (lamivudine/3TC), and COMBIVIR (zidovudine/lamivudine). Non-nucleoside reverse transcriptase inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, VIRAMUNE (nevirapine), RESCRIPTOR (delavirdine), and  
15   SUSTIVA (efavirenz). Protease inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, CRIXIVAN (indinavir), NORVIR (ritonavir), INVIRASE (saquinavir), and VIRACEPT (nelfinavir). In a specific embodiment, antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or  
20   protease inhibitors may be used in any combination with Therapeutics of the invention to treat AIDS and/or to prevent or treat HIV infection.

          In other embodiments, Therapeutics of the invention may be administered in combination with anti-opportunistic infection agents. Anti-opportunistic agents that may be administered in combination with the Therapeutics of the invention, include,  
25   but are not limited to, TRIMETHOPRIM-SULFAMETHOXAZOLE, DAPSONE, PENTAMIDINE, ATOVAQUONE, ISONIAZID, RIFAMPIN, PYRAZINAMIDE, ETHAMBUTOL, RIFABUTIN, CLARITHROMYCIN, AZITHROMYCIN, GANCICLOVIR, FOSCARNET, CIDOFOVIR, FLUCONAZOLE, ITRACONAZOLE, KETOCONAZOLE, ACYCLOVIR, FAMCICLOVIR,  
30   PYRIMETHAMINE, LEUCOVORIN, NEUPOGEN (filgrastim/G-CSF), and LEUKINE (sargramostim/GM-CSF). In a specific embodiment, Therapeutics of the invention are used in any combination with TRIMETHOPRIM-

SULFAMETHOXAZOLE, DAPSONE, PENTAMIDINE, and/or ATOVAQUONE to prophylactically treat or prevent an opportunistic *Pneumocystis carinii* pneumonia infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ISONIAZID, RIFAMPIN, PYRAZINAMIDE, and/or  
5 ETHAMBUTOL to prophylactically treat or prevent an opportunistic *Mycobacterium avium* complex infection. In another specific embodiment, Therapeutics of the invention are used in any combination with RIFABUTIN, CLARITHROMYCIN, and/or AZITHROMYCIN to prophylactically treat or prevent an opportunistic *Mycobacterium tuberculosis* infection. In another specific embodiment, Therapeutics  
10 of the invention are used in any combination with GANCICLOVIR, FOSCARNET, and/or CIDOFOVIR to prophylactically treat or prevent an opportunistic cytomegalovirus infection. In another specific embodiment, Therapeutics of the invention are used in any combination with FLUCONAZOLE, ITRACONAZOLE, and/or KETOCONAZOLE to prophylactically treat or prevent an opportunistic fungal  
15 infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ACYCLOVIR and/or FAMCICOLVIR to prophylactically treat or prevent an opportunistic herpes simplex virus type I and/or type II infection. In another specific embodiment, Therapeutics of the invention are used in any combination with PYRIMETHAMINE and/or LEUCOVORIN to prophylactically  
20 treat or prevent an opportunistic *Toxoplasma gondii* infection. In another specific embodiment, Therapeutics of the invention are used in any combination with LEUCOVORIN and/or NEUPOGEN to prophylactically treat or prevent an opportunistic bacterial infection.

In a further embodiment, the Therapeutics of the invention are administered in  
25 combination with an antiviral agent. Antiviral agents that may be administered with the Therapeutics of the invention include, but are not limited to, acyclovir, ribavirin, amantadine, and remantidine.

In a further embodiment, the Therapeutics of the invention are administered in combination with an antibiotic agent. Antibiotic agents that may be administered with  
30 the Therapeutics of the invention include, but are not limited to, amoxicillin, beta-lactamases, aminoglycosides, beta-lactam (glycopeptide), beta-lactamases, Clindamycin, chloramphenicol, cephalosporins, ciprofloxacin, ciprofloxacin,

erythromycin, fluoroquinolones, macrolides, metronidazole, penicillins, quinolones, rifampin, streptomycin, sulfonamide, tetracyclines, trimethoprim, trimethoprim-sulfamthoxazole, and vancomycin.

5 Conventional nonspecific immunosuppressive agents, that may be administered in combination with the Therapeutics of the invention include, but are not limited to, steroids, cyclosporine, cyclosporine analogs, cyclophosphamide methylprednisone, prednisone, azathioprine, FK-506, 15-deoxyspergualin, and other immunosuppressive agents that act by suppressing the function of responding T cells.

10 In specific embodiments, Therapeutics of the invention are administered in combination with immunosuppressants. Immunosuppressants preparations that may be administered with the Therapeutics of the invention include, but are not limited to, ORTHOCLONE (OKT3), SANDIMMUNE/NEORAL/SANGDYA (cyclosporin), PROGRAF (tacrolimus), CELLCEPT (mycophenolate), Azathioprine, glucocorticosteroids, and RAPAMUNE (sirolimus). In a specific embodiment,  
15 immunosuppressants may be used to prevent rejection of organ or bone marrow transplantation.

In an additional embodiment, Therapeutics of the invention are administered alone or in combination with one or more intravenous immune globulin preparations. Intravenous immune globulin preparations that may be administered with the  
20 Therapeutics of the invention include, but not limited to, GAMMAR, IVEEGAM, SANDOGLOBULIN, GAMMAGARD S/D, and GAMIMUNE. In a specific embodiment, Therapeutics of the invention are administered in combination with intravenous immune globulin preparations in transplantation therapy (e.g., bone marrow transplant).

25 In an additional embodiment, the Therapeutics of the invention are administered alone or in combination with an anti-inflammatory agent. Anti-inflammatory agents that may be administered with the Therapeutics of the invention include, but are not limited to, glucocorticoids and the nonsteroidal anti-inflammatories, aminoarylcarboxylic acid derivatives, arylacetic acid derivatives,  
30 arylbutyric acid derivatives, arylcarboxylic acids, arylpropionic acid derivatives, pyrazoles, pyrazolones, salicylic acid derivatives, thiazinecarboxamides, e-acetamidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid,

amixetrine, bendazac, benzydamine, bucolome, difenpiramide, ditazol, emorfazone, guaiazulene, nabumetone, nimesulide, orgotein, oxaceprol, paranyline, perisoxal, pifoxime, proquazone, proxazole, and tenidap.

In another embodiment, compositions of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents that may be administered with the Therapeutics of the invention include, but are not limited to, antibiotic derivatives (e.g., doxorubicin, bleomycin, daunorubicin, and dactinomycin); antiestrogens (e.g., tamoxifen); antimetabolites (e.g., fluorouracil, 5-FU, methotrexate, floxuridine, interferon alpha-2b, glutamic acid, plicamycin, mercaptopurine, and 6-thioguanine); cytotoxic agents (e.g., carmustine, BCNU, lomustine, CCNU, cytosine arabinoside, cyclophosphamide, estramustine, hydroxyurea, procarbazine, mitomycin, busulfan, cis-platin, and vincristine sulfate); hormones (e.g., medroxyprogesterone, estramustine phosphate sodium, ethinyl estradiol, estradiol, megestrol acetate, methyltestosterone, diethylstilbestrol diphosphate, chlorotrianisene, and testolactone); nitrogen mustard derivatives (e.g., mephallen, chorambucil, mechlorethamine (nitrogen mustard) and thiotepa); steroids and combinations (e.g., bethamethasone sodium phosphate); and others (e.g., dicarbazine, asparaginase, mitotane, vincristine sulfate, vinblastine sulfate, and etoposide).

In a specific embodiment, Therapeutics of the invention are administered in combination with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or any combination of the components of CHOP. In another embodiment, Therapeutics of the invention are administered in combination with Rituximab. In a further embodiment, Therapeutics of the invention are administered with Rituxmab and CHOP, or Rituxmab and any combination of the components of CHOP.

In an additional embodiment, the Therapeutics of the invention are administered in combination with cytokines. Cytokines that may be administered with the Therapeutics of the invention include, but are not limited to, IL2, IL3, IL4, IL5, IL6, IL7, IL10, IL12, IL13, IL15, anti-CD40, CD40L, IFN-gamma and TNF-alpha. In another embodiment, Therapeutics of the invention may be administered with any interleukin, including, but not limited to, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5,

IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, and IL-21.

In an additional embodiment, the Therapeutics of the invention are administered in combination with angiogenic proteins. Angiogenic proteins that may be administered with the Therapeutics of the invention include, but are not limited to, Glioma Derived Growth Factor (GDGF), as disclosed in European Patent Number EP-399816; Platelet Derived Growth Factor-A (PDGF-A), as disclosed in European Patent Number EP-682110; Platelet Derived Growth Factor-B (PDGF-B), as disclosed in European Patent Number EP-282317; Placental Growth Factor (PIGF), as disclosed in International Publication Number WO 92/06194; Placental Growth Factor-2 (PIGF-2), as disclosed in Hauser et al., Growth Factors, 4:259-268 (1993); Vascular Endothelial Growth Factor (VEGF), as disclosed in International Publication Number WO 90/13649; Vascular Endothelial Growth Factor-A (VEGF-A), as disclosed in European Patent Number EP-506477; Vascular Endothelial Growth Factor-2 (VEGF-2), as disclosed in International Publication Number WO 96/39515; Vascular Endothelial Growth Factor B (VEGF-3); Vascular Endothelial Growth Factor B-186 (VEGF-B186), as disclosed in International Publication Number WO 96/26736; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/02543; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/07832; and Vascular Endothelial Growth Factor-E (VEGF-E), as disclosed in German Patent Number DE19639601. The above mentioned references are incorporated herein by reference herein.

In an additional embodiment, the Therapeutics of the invention are administered in combination with hematopoietic tubulin tyrosine ligase proteins. Hematopoietic tubulin tyrosine ligase proteins that may be administered with the Therapeutics of the invention include, but are not limited to, LEUKINE (SARGRAMOSTIM) and NEUPOGEN (FILGRASTIM).

In an additional embodiment, the Therapeutics of the invention are administered in combination with Fibroblast Growth Factors. Fibroblast Growth Factors that may be administered with the Therapeutics of the invention include, but

are not limited to, FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, and FGF-15.

In a specific embodiment, formulations of the present invention may further comprise antagonists of P-glycoprotein (also referred to as the multiresistance protein, or PGP), including antagonists of its encoding polynucleotides (e.g., antisense oligonucleotides, ribozymes, zinc-finger proteins, etc.). P-glycoprotein is well known for decreasing the efficacy of various drug administrations due to its ability to export intracellular levels of absorbed drug to the cell exterior. While this activity has been particularly pronounced in cancer cells in response to the administration of chemotherapy regimens, a variety of other cell types and the administration of other drug classes have been noted (e.g., T-cells and anti-HIV drugs). In fact, certain mutations in the PGP gene significantly reduces PGP function, making it less able to force drugs out of cells. People who have two versions of the mutated gene--one inherited from each parent--have more than four times less PGP than those with two normal versions of the gene. People may also have one normal gene and one mutated one. Certain ethnic populations have increased incidence of such PGP mutations. Among individuals from Ghana, Kenya, the Sudan, as well as African Americans, frequency of the normal gene ranged from 73% to 84%. In contrast, the frequency was 34% to 59% among British whites, Portuguese, Southwest Asian, Chinese, Filipino and Saudi populations. As a result, certain ethnic populations may require increased administration of PGP antagonist in the formulation of the present invention to arrive at the an efficacious dose of the therapeutic (e.g., those from African descent). Conversely, certain ethnic populations, particularly those having increased frequency of the mutated PGP (e.g., of Caucasian descent, or non-African descent) may require less pharmaceutical compositions in the formulation due to an effective increase in efficacy of such compositions as a result of the increased effective absorption (e.g., less PGP activity) of said composition.

Moreover, in another specific embodiment, formulations of the present invention may further comprise antagonists of OATP2 (also referred to as the multiresistance protein, or MRP2), including antagonists of its encoding polynucleotides (e.g., antisense oligonucleotides, ribozymes, zinc-finger proteins, etc.). The invention also further comprises any additional antagonists known to inhibit



proteins thought to be attributable to a multidrug resistant phenotype in proliferating cells.

Preferred antagonists that formulations of the present may comprise include the potent P-glycoprotein inhibitor elacridar, and/or LY-335979. Other P-glycoprotein 5 inhibitors known in the art are also encompassed by the present invention.

In additional embodiments, the Therapeutics of the invention are administered in combination with other therapeutic or prophylactic regimens, such as, for example, radiation therapy.

**EXAMPLE 26 – METHOD OF TREATING DECREASED LEVELS  
OF THE POLYPEPTIDE**

The present invention relates to a method for treating an individual in need of an increased level of a polypeptide of the invention in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of an agonist of the invention (including polypeptides of the invention). Moreover, it will be appreciated that conditions caused by a decrease in the standard or normal expression level of a secreted protein in an individual can be treated by administering the polypeptide of the present invention, preferably in the secreted form. Thus, the invention also provides a method of treatment of an individual in need of an increased level of the polypeptide comprising administering to such an individual a Therapeutic comprising an amount of the polypeptide to increase the activity level of the polypeptide in such an individual.

For example, a patient with decreased levels of a polypeptide receives a daily dose 0.1-100 ug/kg of the polypeptide for six consecutive days. Preferably, the polypeptide is in the secreted form. The exact details of the dosing scheme, based on administration and formulation, are provided herein.

### EXAMPLE 27 – METHOD OF TREATING INCREASED LEVELS OF THE POLYPEPTIDE

30           The present invention also relates to a method of treating an individual in need of a decreased level of a polypeptide of the invention in the body comprising administering to such an individual a composition comprising a therapeutically

effective amount of an antagonist of the invention (including polypeptides and antibodies of the invention).

In one example, antisense technology is used to inhibit production of a polypeptide of the present invention. This technology is one example of a method of decreasing levels of a polypeptide, preferably a secreted form, due to a variety of etiologies, such as cancer. For example, a patient diagnosed with abnormally increased levels of a polypeptide is administered intravenously antisense polynucleotides at 0.5, 1.0, 1.5, 2.0 and 3.0 mg/kg day for 21 days. This treatment is repeated after a 7-day rest period if the treatment was well tolerated. The formulation of the antisense polynucleotide is provided herein.

#### EXAMPLE 28 – METHOD OF TREATMENT USING GENE THERAPY-EX VIVO

One method of gene therapy transplants fibroblasts, which are capable of expressing a polypeptide, onto a patient. Generally, fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37 degree C for approximately one week.

At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention can be amplified using PCR primers which correspond to the 5' and 3' end sequences respectively as set forth in Example 13 using primers and having appropriate restriction sites and

initiation/stop codons, if necessary. Preferably, the 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is then used to transform bacteria HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector has the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether protein is produced.

The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

#### EXAMPLE 29 – GENE THERAPY USING ENDOGENOUS GENES

#### CORRESPONDING TO POLYNUCLEOTIDES OF THE INVENTION

Another method of gene therapy according to the present invention involves operably associating the endogenous polynucleotide sequence of the invention with a

promoter via homologous recombination as described, for example, in U.S. Patent NO: 5,641,670, issued June 24, 1997; International Publication NO: WO 96/29411, published September 26, 1996; International Publication NO: WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA, 86:8932-8935 (1989); and Zijlstra et al., Nature, 342:435-438 (1989). This method involves the activation of a gene which is present in the target cells, but which is not expressed in the cells, or is expressed at a lower level than desired.

Polynucleotide constructs are made which contain a promoter and targeting sequences, which are homologous to the 5' non-coding sequence of endogenous polynucleotide sequence, flanking the promoter. The targeting sequence will be sufficiently near the 5' end of the polynucleotide sequence so the promoter will be operably linked to the endogenous sequence upon homologous recombination. The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter.

The amplified promoter and the amplified targeting sequences are digested with the appropriate restriction enzymes and subsequently treated with calf intestinal phosphatase. The digested promoter and digested targeting sequences are added together in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The construct is size fractionated on an agarose gel then purified by phenol extraction and ethanol precipitation.

In this Example, the polynucleotide constructs are administered as naked polynucleotides via electroporation. However, the polynucleotide constructs may also be administered with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, precipitating agents, etc. Such methods of delivery are known in the art.

Once the cells are transfected, homologous recombination will take place which results in the promoter being operably linked to the endogenous polynucleotide

sequence. This results in the expression of polynucleotide corresponding to the polynucleotide in the cell. Expression may be detected by immunological staining, or any other method known in the art.

5 Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in DMEM + 10% fetal calf serum. Exponentially growing or early stationary phase fibroblasts are trypsinized and rinsed from the plastic surface with nutrient medium. An aliquot of the cell suspension is removed for counting, and the remaining cells are subjected to centrifugation. The supernatant is aspirated and the pellet is resuspended in 5 ml of electroporation buffer (20 mM HEPES pH 7.3, 137 mM NaCl,  
10 5 mM KCl, 0.7 mM Na<sub>2</sub> HPO<sub>4</sub>, 6 mM dextrose). The cells are recentrifuged, the supernatant aspirated, and the cells resuspended in electroporation buffer containing 1 mg/ml acetylated bovine serum albumin. The final cell suspension contains approximately 3X10<sup>6</sup> cells/ml. Electroporation should be performed immediately following resuspension.

15 Plasmid DNA is prepared according to standard techniques. For example, to construct a plasmid for targeting to the locus corresponding to the polynucleotide of the invention, plasmid pUC18 (MBI Fermentas, Amherst, NY) is digested with HindIII. The CMV promoter is amplified by PCR with an XbaI site on the 5' end and a BamHI site on the 3'end. Two non-coding sequences are amplified via PCR: one  
20 non-coding sequence (fragment 1) is amplified with a HindIII site at the 5' end and an Xba site at the 3'end; the other non-coding sequence (fragment 2) is amplified with a BamHI site at the 5'end and a HindIII site at the 3'end. The CMV promoter and the fragments (1 and 2) are digested with the appropriate enzymes (CMV promoter - XbaI and BamHI; fragment 1 - XbaI; fragment 2 - BamHI) and ligated together. The  
25 resulting ligation product is digested with HindIII, and ligated with the HindIII-digested pUC18 plasmid.

Plasmid DNA is added to a sterile cuvette with a 0.4 cm electrode gap (Bio-Rad). The final DNA concentration is generally at least 120 µg/ml. 0.5 ml of the cell suspension (containing approximately 1.5X10<sup>6</sup> cells) is then added to the cuvette,  
30 and the cell suspension and DNA solutions are gently mixed. Electroporation is performed with a Gene-Pulser apparatus (Bio-Rad). Capacitance and voltage are set at 960 µF and 250-300 V, respectively. As voltage increases, cell survival decreases, but

the percentage of surviving cells that stably incorporate the introduced DNA into their genome increases dramatically. Given these parameters, a pulse time of approximately 14-20 mSec should be observed.

Electroporated cells are maintained at room temperature for approximately 5 min, and the contents of the cuvette are then gently removed with a sterile transfer pipette. The cells are added directly to 10 ml of prewarmed nutrient media (DMEM with 15% calf serum) in a 10 cm dish and incubated at 37 degree C. The following day, the media is aspirated and replaced with 10 ml of fresh media and incubated for a further 16-24 hours.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product. The fibroblasts can then be introduced into a patient as described above.

#### EXAMPLE 30 – METHOD OF TREATMENT USING GENE THERAPY - IN VIVO

Another aspect of the present invention is using in vivo gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) sequences into an animal to increase or decrease the expression of the polypeptide. The polynucleotide of the present invention may be operatively linked to a promoter or any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO. 5693622, 5705151, 5580859; Tabata et al., Cardiovasc. Res. 35(3):470-479 (1997); Chao et al., Pharmacol. Res. 35(6):517-522 (1997); Wolff, Neuromuscul. Disord. 7(5):314-318 (1997); Schwartz et al., Gene Ther. 3(5):405-411 (1996); Tsurumi et al., Circulation 94(12):3281-3290 (1996) (incorporated herein by reference).

The polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The

polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

5 The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the polynucleotides of the present invention may also be delivered in liposome formulations (such as those taught in Felgner P.L. et al. (1995) Ann. NY Acad. Sci. 772:126-139 and Abdallah B. et al. (1995) Biol. Cell 85(1):1-7) which can be prepared by methods well known to those skilled in the art.

10 The polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapies techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

15 The polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated

cells, such as, for example, stem cells of blood or skin fibroblasts. In vivo muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The dose response effects of injected polynucleotide in muscle in vivo is determined as follows. Suitable template DNA for production of mRNA coding for polypeptide of the present invention is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for protein expression. A time course for protein expression may be done in a similar fashion except that



quadriceps from different mice are harvested at different times. Persistence of DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be use to extrapolate proper dosages and other treatment parameters in humans and other animals using naked DNA.

#### EXAMPLE 31 – TRANSGENIC ANIMALS

The polypeptides of the invention can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express polypeptides of the invention in humans, as part of a gene therapy protocol.

Any technique known in the art may be used to introduce the transgene (i.e., polynucleotides of the invention) into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson et al., Appl. Microbiol. Biotechnol. 40:691-698 (1994); Carver et al., Biotechnology (NY) 11:1263-1270 (1993); Wright et al., Biotechnology (NY) 9:830-834 (1991); and Hoppe et al., U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson et al., Cell 56:313-321 (1989)); electroporation of cells or embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, e.g., Ulmer et al., Science 259:1745 (1993); introducing nucleic acid constructs into embryonic pluripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer (Lavitrano et al., Cell 57:717-723 (1989); etc. For a review of such techniques, see Gordon, "Transgenic Animals" Intl. Rev. Cytol. 115:171-229 (1989), which is incorporated by reference herein in its entirety.

Any technique known in the art may be used to produce transgenic clones containing polynucleotides of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence (Campell et al., *Nature* 380:64-66 (1996); Wilmut et al., *Nature* 385:810-813 (1997)).

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals or chimeric. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko et al., *Proc. Natl. Acad. Sci. USA* 89:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., *Science* 265:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and reverse

transcriptase-PCR(RT-PCR).. Samples of transgenic gene-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the transgene product.

Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and breeding to place the transgene on a distinct background that is appropriate for an experimental model of interest.

Transgenic animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of polypeptides of the present invention, studying diseases, disorders, and/or conditions associated with aberrant expression, and in screening for compounds effective in ameliorating such diseases, disorders, and/or conditions.

#### EXAMPLE 32 – KNOCK-OUT ANIMALS

Endogenous gene expression can also be reduced by inactivating or "knocking out" the gene and/or its promoter using targeted homologous recombination. (E.g., see Smithies et al., Nature 317:230-234 (1985); Thomas & Capecchi, Cell 51:503-512 (1987); Thompson et al., Cell 5:313-321 (1989); each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional polynucleotide of the invention (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous polynucleotide sequence (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express polypeptides of the invention in vivo. In another embodiment, techniques known in the art are used to

generate knockouts in cells that contain, but do not express the gene of interest. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the targeted gene. Such approaches are particularly suited in research and agricultural fields where modifications to embryonic stem cells can be used to generate animal offspring with an inactive targeted gene (e.g., see Thomas & Capecchi 1987 and Thompson 1989, supra). However this approach can be routinely adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site in vivo using appropriate viral vectors that will be apparent to those of skill in the art.

10 In further embodiments of the invention, cells that are genetically engineered to express the polypeptides of the invention, or alternatively, that are genetically engineered not to express the polypeptides of the invention (e.g., knockouts) are administered to a patient in vivo. Such cells may be obtained from the patient (i.e., animal, including human) or an MHC compatible donor and can include, but are not limited to fibroblasts, bone marrow cells, blood cells (e.g., lymphocytes), adipocytes, muscle cells, endothelial cells etc. The cells are genetically engineered in vitro using recombinant DNA techniques to introduce the coding sequence of polypeptides of the invention into the cells, or alternatively, to disrupt the coding sequence and/or endogenous regulatory sequence associated with the polypeptides of the invention, e.g., by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, naked DNA, electroporation, liposomes, etc. The coding sequence of the polypeptides of the invention can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve expression, and preferably secretion, of the polypeptides of the invention. The engineered cells which express and preferably secrete the polypeptides of the invention can be introduced into the patient systemically, e.g., in the circulation, or intraperitoneally.

Alternatively, the cells can be incorporated into a matrix and implanted in the body, e.g., genetically engineered fibroblasts can be implanted as part of a skin graft; genetically engineered endothelial cells can be implanted as part of a lymphatic or vascular graft. (See, for example, Anderson et al. U.S. Patent No. 5,399,349; and

Mulligan & Wilson, U.S. Patent No. 5,460,959 each of which is incorporated by reference herein in its entirety).

When the cells to be administered are non-autologous or non-MHC compatible cells, they can be administered using well known techniques which prevent the development of a host immune response against the introduced cells. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

Transgenic and "knock-out" animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of polypeptides of the present invention, studying diseases, disorders, and/or conditions associated with aberrant expression, and in screening for compounds effective in ameliorating such diseases, disorders, and/or conditions.

#### EXAMPLE 33 – METHOD OF ISOLATING ANTIBODY FRAGMENTS DIRECTED AGAINST BGS-42 FROM A LIBRARY OF scFvs

Naturally occurring V-genes isolated from human PBLs are constructed into a library of antibody fragments which contain reactivities against BGS-42 to which the donor may or may not have been exposed (see e.g., U.S. Patent 5,885,793 incorporated herein by reference in its entirety).

Rescue of the Library. A library of scFvs is constructed from the RNA of human PBLs as described in PCT publication WO 92/01047. To rescue phage displaying antibody fragments, approximately 10<sup>9</sup> E. coli harboring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100 µg/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to inoculate 50 ml of 2xTY-AMP-GLU, 2 x 10<sup>8</sup> TU of delta gene 3 helper (M13 delta gene III, see PCT publication WO 92/01047) are added and the culture incubated at 37°C for 45 minutes without shaking and then at 37°C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100 µg/ml ampicillin and 50 ug/ml kanamycin and grown overnight. Phage are prepared as described in PCT publication WO 92/01047.

M13 delta gene III is prepared as follows: M13 delta gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13 delta gene III particles are made by growing the helper phage in cells harboring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37° C without shaking and then for a further hour at 37°C with shaking. Cells are spun down (IEC-Centra 8,400 r.p.m. for 10 min), resuspended in 300 ml 2xTY broth containing 100 µg ampicillin/ml and 25 µg kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37°C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook et al., 1990), resuspended in 2 ml PBS and passed through a 0.45 µm filter (Minisart NML; Sartorius) to give a final concentration of approximately 10<sup>13</sup> transducing units/ml (ampicillin-resistant clones).

Panning of the Library. Immuntubes (Nunc) are coated overnight in PBS with 4 ml of either 100 µg/ml or 10 µg/ml of a polypeptide of the present invention. Tubes are blocked with 2% Marvel-PBS for 2 hours at 37°C and then washed 3 times in PBS. Approximately 10<sup>13</sup> TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log E. coli TG1 by incubating eluted phage with bacteria for 30 minutes at 37°C. The E. coli are then plated on TYE plates containing 1% glucose and 100 µg/ml ampicillin. The resulting bacterial library is then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Characterization of Binders. Eluted phage from the 3rd and 4th rounds of selection are used to infect E. coli HB 2151 and soluble scFv is produced (Marks, et al., 1991) from single colonies for assay. ELISAs are performed with microtitre plates

coated with either 10 pg/ml of the polypeptide of the present invention in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see, e.g., PCT publication WO 92/01047) and then by sequencing. These ELISA positive clones may also be further characterized by techniques known  
5 in the art, such as, for example, epitope mapping, binding affinity, receptor signal transduction, ability to block or competitively inhibit antibody/antigen binding, and competitive agonistic or antagonistic activity.

Moreover, in another preferred method, the antibodies directed against the polypeptides of the present invention may be produced in plants. Specific methods are  
10 disclosed in US Patent Nos. 5,959,177, and 6,080,560, which are hereby incorporated in their entirety herein. The methods not only describe methods of expressing antibodies, but also the means of assembling foreign multimeric proteins in plants (i.e., antibodies, etc.), and the subsequent secretion of such antibodies from the plant.

#### 15 EXAMPLE 34 – IDENTIFICATION AND CLONING OF VH AND VL DOMAINS OF ANTIBODIES DIRECTED AGAINST THE BGS-42 POLYPEPTIDE

VH and VL domains may be identified and cloned from cell lines expressing an antibody directed against a BGS-42 epitope by performing PCR with VH and VL specific primers on cDNA made from the antibody expressing cell lines. Briefly,  
20 RNA is isolated from the cell lines and used as a template for RT-PCR designed to amplify the VH and VL domains of the antibodies expressed by the EBV cell lines. Cells may be lysed using the TRIzol reagent (Life Technologies, Rockville, MD) and extracted with one fifth volume of chloroform. After addition of chloroform, the solution is allowed to incubate at room temperature for 10 minutes, and then  
25 centrifuged at 14, 000 rpm for 15 minutes at 4 C in a tabletop centrifuge. The supernatant is collected and RNA is precipitated using an equal volume of isopropanol. Precipitated RNA is pelleted by centrifuging at 14, 000 rpm for 15 minutes at 4 C in a tabletop centrifuge.

Following centrifugation, the supernatant is discarded and washed with 75%  
30 ethanol. Following the wash step, the RNA is centrifuged again at 800 rpm for 5 minutes at 4 C. The supernatant is discarded and the pellet allowed to air dry. RNA is dissolved in DEPC water and heated to 60 C for 10 minutes. Quantities of RNA

can be determined using optical density measurements. CDNA may be synthesized, according to methods well-known in the art and/or described herein, from 1. 5-2. 5 micrograms of RNA using reverse transcriptase and random hexamer primers. CDNA is then used as a template for PCR amplification of VH and VL domains.

5 Primers used to amplify VH and VL genes are shown below. Typically a PCR reaction makes use of a single 5'primer and a single 3'primer. Sometimes, when the amount of available RNA template is limiting, or for greater efficiency, groups of 5' and/or 3'primers may be used. For example, sometimes all five VH-5'primers and all JH3'primers are used in a single PCR reaction. The PCR reaction is carried out in a  
10 50 microliter volume containing 1X PCR buffer, 2mM of each dNTP, 0. 7 units of High Fidelity Taq polymerase, 5'primer mix, 3'primer mix and 7. 5 microliters of cDNA. The 5'and 3'primer mix of both VH and VL can be made by pooling together 22 pmole and 28 pmole, respectively, of each of the individual primers. PCR conditions are : 96 C for 5 minutes ; followed by 25 cycles of 94 C for 1 minute, 50 C  
15 for 1 minute, and 72 C for 1 minute ; followed by an extension cycle of 72 C for 10 minutes. After the reaction has been completed, sample tubes may be stored at 4 C.

Primer Sequences Used to Amplify VH domains

Primer name	Primer Sequence	SEQ ID NO:
Hu VH1 – 5'	CAGGTGCAGCTGGTGCAGTCTGG	67
Hu VH2 – 5'	CAGGTCAACTTAAGGGAGTCTGG	68
Hu VH3 – 5'	GAGGTGCAGCTGGTGGAGTCTGG	69
Hu VH4 – 5'	CAGGTGCAGCTGCAGGAGTCGGG	70
Hu VH5 – 5'	GAGGTGCAGCTGTTGCAGTCTGC	71
Hu VH6 – 5'	CAGGTACAGCTGCAGCAGTCAGG	72
Hu JH1 – 5'	TGAGGAGACGGTGACCAGGGTGCC	73
Hu JH3 – 5'	TGAAGAGACGGTGACCATTTGTCCC	74
Hu JH4 – 5'	TGAGGAGACGGTGACCAGGGTTCC	75
Hu JH6 – 5'	TGAGGAGACGGTGACCGTGGTCCC	76

20

Primer Sequences Used to Amplify VL domains

Primer name	Primer Sequence	SEQ ID NO:
Hu Vkappa1 – 5'	GACATCCAGATGACCCAGTCTCC	77
Hu Vkappa2a – 5'	GATGTTGTGATGACTCAGTCTCC	78
Hu Vkappa2b – 5'	GATATTGTGATGACTCAGTCTCC	79
Hu Vkappa3 – 5'	GAAATTGTGTTGACGCAGTCTCC	80
Hu Vkappa4 – 5'	GACATCGTGATGACCCAGTCTCC	81



Primer name	Primer Sequence	SEQ ID NO:
Hu Vkappa5 – 5'	GAAACGACACTCACGCAGTCTCC	82
Hu Vkappa6 – 5'	GAAATTGTGCTGACTCAGTCTCC	83
Hu Vlambdal – 5'	CAGTCTGTGTTGACGCAGCCGCC	84
Hu Vlambda2 – 5'	CAGTCTGCCCTGACTCAGCCTGC	85
Hu Vlambda3 – 5'	TCCTATGTGCTGACTCAGCCACC	86
Hu Vlambda3b – 5'	TCTTCTGAGCTGACTCAGGACCC	87
Hu Vlambda4 – 5'	CACGTTATACTGACTCAACCGCC	88
Hu Vlambda5 – 5'	CAGGCTGTGCTCACTCAGCCGTC	89
Hu Vlambda6 – 5'	AATTTTATGCTGACTCAGCCCCA	90
Hu Jkappa1 – 3'	ACGTTTGATTTCACCTTGGTCCC	91
Hu Jkappa2 – 3'	ACGTTTGATCTCCAGCTTGGTCCC	92
Hu Jkappa3 – 3'	ACGTTTGATATCCACTTTGGTCCC	93
Hu Jkappa4 – 3'	ACGTTTGATCTCCACCTTGGTCCC	94
Hu Jkappa5 – 3'	ACGTTTAATCTCCAGTCGTGTCCC	95
Hu Vlambdal – 3'	CAGTCTGTGTTGACGCAGCCGCC	96
Hu Vlambda2 – 3'	CAGTCTGCCCTGACTCAGCCTGC	97
Hu Vlambda3 – 3'	TCCTATGTGCTGACTCAGCCACC	98
Hu Vlambda3b – 3'	TCTTCTGAGCTGACTCAGGACCC	99
Hu Vlambda4 – 3'	CACGTTATACTGACTCAACCGCC	100
Hu Vlambda5 – 3'	CAGGCTGTGCTCACTCAGCCGTC	101
Hu Vlambda6 – 3'	AATTTTATGCTGACTCAGCCCCA	102

PCR samples are then electrophoresed on a 1.3% agarose gel. DNA bands of the expected sizes (~506 base pairs for VH domains, and 344 base pairs for VL domains) can be cut out of the gel and purified using methods well known in the art and/or described herein.

Purified PCR products can be ligated into a PCR cloning vector (TA vector from Invitrogen Inc., Carlsbad, CA). Individual cloned PCR products can be isolated after transfection of E. coli and blue/white color selection. Cloned PCR products may then be sequenced using methods commonly known in the art and/or described herein.

The PCR bands containing the VH domain and the VL domains can also be used to create full-length Ig expression vectors. VH and VL domains can be cloned into vectors containing the nucleotide sequences of a heavy (e. g., human IgG1 or human IgG4) or light chain (human kappa or human lambda) constant regions such that a complete heavy or light chain molecule could be expressed from these vectors when transfected into an appropriate host cell. Further, when cloned heavy and light chains are both expressed in one cell line (from either one or two vectors), they can assemble into a complete functional antibody molecule that is secreted into the cell

culture medium. Methods using polynucleotides encoding VH and VL antibody domain to generate expression vectors that encode complete antibody molecules are well known within the art.

5           **EXAMPLE 35 – ASSAYS DETECTING STIMULATION OR INHIBITION  
                  OF B CELL PROLIFERATION AND DIFFERENTIATION**

          Generation of functional humoral immune responses requires both soluble and cognate signaling between B-lineage cells and their microenvironment. Signals may impart a positive stimulus that allows a B-lineage cell to continue its programmed  
10   development, or a negative stimulus that instructs the cell to arrest its current developmental pathway. To date, numerous stimulatory and inhibitory signals have been found to influence B cell responsiveness including IL-2, IL-4, IL-5, IL-6, IL-7, IL10, IL-13, IL-14 and IL-15. Interestingly, these signals are by themselves weak effectors but can, in combination with various co-stimulatory proteins, induce  
15   activation, proliferation, differentiation, homing, tolerance and death among B cell populations.

          One of the best studied classes of B-cell co-stimulatory proteins is the TNF-superfamily. Within this family CD40, CD27, and CD30 along with their respective ligands CD154, CD70, and CD153 have been found to regulate a variety of immune  
20   responses. Assays which allow for the detection and/or observation of the proliferation and differentiation of these B-cell populations and their precursors are valuable tools in determining the effects various proteins may have on these B-cell populations in terms of proliferation and differentiation. Listed below are two assays designed to allow for the detection of the differentiation, proliferation, or inhibition of  
25   B-cell populations and their precursors.

          In Vitro Assay- Purified polypeptides of the invention, or truncated forms thereof, is assessed for its ability to induce activation, proliferation, differentiation or inhibition and/or death in B-cell populations and their precursors. The activity of the polypeptides of the invention on purified human tonsillar B cells, measured  
30   qualitatively over the dose range from 0.1 to 10,000 ng/mL, is assessed in a standard B-lymphocyte co-stimulation assay in which purified tonsillar B cells are cultured in the presence of either formalin-fixed *Staphylococcus aureus* Cowan I (SAC) or

immobilized anti-human IgM antibody as the priming agent. Second signals such as IL-2 and IL-15 synergize with SAC and IgM crosslinking to elicit B cell proliferation as measured by tritiated-thymidine incorporation. Novel synergizing agents can be readily identified using this assay. The assay involves isolating human tonsillar B  
5 cells by magnetic bead (MACS) depletion of CD3-positive cells. The resulting cell population is greater than 95% B cells as assessed by expression of CD45R(B220).

Various dilutions of each sample are placed into individual wells of a 96-well plate to which are added 10<sup>5</sup> B-cells suspended in culture medium (RPMI 1640 containing 10% FBS, 5 X 10<sup>-5</sup>M 2ME, 100U/ml penicillin, 10ug/ml streptomycin,  
10 and 10<sup>-5</sup> dilution of SAC) in a total volume of 150ul. Proliferation or inhibition is quantitated by a 20h pulse (1uCi/well) with 3H-thymidine (6.7 Ci/mM) beginning 72h post factor addition. The positive and negative controls are IL2 and medium respectively.

In Vivo Assay- BALB/c mice are injected (i.p.) twice per day with buffer  
15 only, or 2 mg/Kg of a polypeptide of the invention, or truncated forms thereof. Mice receive this treatment for 4 consecutive days, at which time they are sacrificed and various tissues and serum collected for analyses. Comparison of H&E sections from normal spleens and spleens treated with polypeptides of the invention identify the results of the activity of the polypeptides on spleen cells, such as the diffusion of peri-  
20 arterial lymphatic sheaths, and/or significant increases in the nucleated cellularity of the red pulp regions, which may indicate the activation of the differentiation and proliferation of B-cell populations. Immunohistochemical studies using a B cell marker, anti-CD45R(B220), are used to determine whether any physiological changes to splenic cells, such as splenic disorganization, are due to increased B-cell  
25 representation within loosely defined B-cell zones that infiltrate established T-cell regions.

Flow cytometric analyses of the spleens from mice treated with polypeptide is used to indicate whether the polypeptide specifically increases the proportion of ThB+, CD45R(B220)dull B cells over that which is observed in control mice.

30 Likewise, a predicted consequence of increased mature B-cell representation in vivo is a relative increase in serum Ig titers. Accordingly, serum IgM and IgA levels are compared between buffer and polypeptide-treated mice.

One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

5                                   EXAMPLE 36 – T CELL PROLIFERATION ASSAY

A CD3-induced proliferation assay is performed on PBMCs and is measured by the uptake of 3H-thymidine. The assay is performed as follows. Ninety-six well plates are coated with 100 (l/well of mAb to CD3 (HIT3a, Pharmingen) or isotype-matched control mAb (B33.1) overnight at 4 degrees C (1 (g/ml in .05M bicarbonate  
10 buffer, pH 9.5), then washed three times with PBS. PBMC are isolated by F/H gradient centrifugation from human peripheral blood and added to quadruplicate wells (5 x 10<sup>4</sup>/well) of mAb coated plates in RPMI containing 10% FCS and P/S in the presence of varying concentrations of polypeptides of the invention (total volume 200 ul). Relevant protein buffer and medium alone are controls. After 48 hr. culture at 37  
15 degrees C, plates are spun for 2 min. at 1000 rpm and 100 (l of supernatant is removed and stored –20 degrees C for measurement of IL-2 (or other cytokines) if effect on proliferation is observed. Wells are supplemented with 100 ul of medium containing 0.5 uCi of 3H-thymidine and cultured at 37 degrees C for 18-24 hr. Wells are harvested and incorporation of 3H-thymidine used as a measure of proliferation.  
20 Anti-CD3 alone is the positive control for proliferation. IL-2 (100 U/ml) is also used as a control which enhances proliferation. Control antibody which does not induce proliferation of T cells is used as the negative controls for the effects of polypeptides of the invention.

One skilled in the art could easily modify the exemplified studies to test the  
25 activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

EXAMPLE 37 – EFFECT OF POLYPEPTIDES OF THE INVENTION ON THE  
EXPRESSION OF MHC CLASS II, COSTIMULATORY AND ADHESION  
MOLECULES AND CELL DIFFERENTIATION OF MONOCYTES AND  
MONOCYTE-DERIVED HUMAN DENDRITIC CELLS

5 Dendritic cells are generated by the expansion of proliferating precursors found in the peripheral blood: adherent PBMC or elutriated monocytic fractions are cultured for 7-10 days with GM-CSF (50 ng/ml) and IL-4 (20 ng/ml). These dendritic cells have the characteristic phenotype of immature cells (expression of CD1, CD80, CD86, CD40 and MHC class II antigens). Treatment with activating factors, such as  
10 TNF-, causes a rapid change in surface phenotype (increased expression of MHC class I and II, costimulatory and adhesion molecules, downregulation of FC(RII, upregulation of CD83). These changes correlate with increased antigen-presenting capacity and with functional maturation of the dendritic cells.

FACS analysis of surface antigens is performed as follows. Cells are treated 1-  
15 3 days with increasing concentrations of polypeptides of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4 degrees C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

20 Effect on the production of cytokines. Cytokines generated by dendritic cells, in particular IL-12, are important in the initiation of T-cell dependent immune responses. IL-12 strongly influences the development of Th1 helper T-cell immune response, and induces cytotoxic T and NK cell function. An ELISA is used to measure the IL-12 release as follows. Dendritic cells (10<sup>6</sup>/ml) are treated with  
25 increasing concentrations of polypeptides of the invention for 24 hours. LPS (100 ng/ml) is added to the cell culture as positive control. Supernatants from the cell cultures are then collected and analyzed for IL-12 content using commercial ELISA kit(e.g., R & D Systems (Minneapolis, MN)). The standard protocols provided with the kits are used.

30 Effect on the expression of MHC Class II, costimulatory and adhesion molecules. Three major families of cell surface antigens can be identified on monocytes: adhesion molecules, molecules involved in antigen presentation, and Fc

receptor. Modulation of the expression of MHC class II antigens and other costimulatory molecules, such as B7 and ICAM-1, may result in changes in the antigen presenting capacity of monocytes and ability to induce T cell activation. Increase expression of Fc receptors may correlate with improved monocyte cytotoxic activity, cytokine release and phagocytosis.

FACS analysis is used to examine the surface antigens as follows. Monocytes are treated 1-5 days with increasing concentrations of polypeptides of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4 degrees C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

Monocyte activation and/or increased survival. Assays for molecules that activate (or alternatively, inactivate) monocytes and/or increase monocyte survival (or alternatively, decrease monocyte survival) are known in the art and may routinely be applied to determine whether a molecule of the invention functions as an inhibitor or activator of monocytes. Polypeptides, agonists, or antagonists of the invention can be screened using the three assays described below. For each of these assays, Peripheral blood mononuclear cells (PBMC) are purified from single donor leukopacks (American Red Cross, Baltimore, MD) by centrifugation through a Histopaque gradient (Sigma). Monocytes are isolated from PBMC by counterflow centrifugal elutriation.

Monocyte Survival Assay. Human peripheral blood monocytes progressively lose viability when cultured in absence of serum or other stimuli. Their death results from internally regulated process (apoptosis). Addition to the culture of activating factors, such as TNF-alpha dramatically improves cell survival and prevents DNA fragmentation. Propidium iodide (PI) staining is used to measure apoptosis as follows. Monocytes are cultured for 48 hours in polypropylene tubes in serum-free medium (positive control), in the presence of 100 ng/ml TNF-alpha (negative control), and in the presence of varying concentrations of the compound to be tested. Cells are suspended at a concentration of  $2 \times 10^6$ /ml in PBS containing PI at a final concentration of 5 (g/ml, and then incubated at room temperature for 5 minutes before

FACScan analysis. PI uptake has been demonstrated to correlate with DNA fragmentation in this experimental paradigm.

Effect on cytokine release. An important function of monocytes/macrophages is their regulatory activity on other cellular populations of the immune system through the release of cytokines after stimulation. An ELISA to measure cytokine release is performed as follows. Human monocytes are incubated at a density of  $5 \times 10^5$  cells/ml with increasing concentrations of the a polypeptide of the invention and under the same conditions, but in the absence of the polypeptide. For IL-12 production, the cells are primed overnight with IFN (100 U/ml) in presence of a polypeptide of the invention. LPS (10 ng/ml) is then added. Conditioned media are collected after 24h and kept frozen until use. Measurement of TNF-alpha, IL-10, MCP-1 and IL-8 is then performed using a commercially available ELISA kit(e.g., R & D Systems (Minneapolis, MN)) and applying the standard protocols provided with the kit.

Oxidative burst. Purified monocytes are plated in 96-w plate at  $2 \times 10^5$  cell/well. Increasing concentrations of polypeptides of the invention are added to the wells in a total volume of 0.2 ml culture medium (RPMI 1640 + 10% FCS, glutamine and antibiotics). After 3 days incubation, the plates are centrifuged and the medium is removed from the wells. To the macrophage monolayers, 0.2 ml per well of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 19 U/ml of HRPO) is added, together with the stimulant (200 nM PMA). The plates are incubated at 37°C for 2 hours and the reaction is stopped by adding 20  $\mu$ l 1N NaOH per well. The absorbance is read at 610 nm. To calculate the amount of H<sub>2</sub>O<sub>2</sub> produced by the macrophages, a standard curve of a H<sub>2</sub>O<sub>2</sub> solution of known molarity is performed for each experiment.

One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

#### EXAMPLE 38 – THE EFFECT OF THE BGS-42 POLYPEPTIDES OF THE INVENTION ON THE GROWTH OF VASCULAR ENDOTHELIAL CELLS

On day 1, human umbilical vein endothelial cells (HUVEC) are seeded at  $2 \times 10^4$  cells/35 mm dish density in M199 medium containing 4% fetal bovine serum

(FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnology, Inc.). On day 2, the medium is replaced with M199 containing 10% FBS, 8 units/ml heparin. A polypeptide having the amino acid sequence of SEQ ID NO:2, and positive controls, such as VEGF and basic FGF (bFGF) are added, at  
5 varying concentrations. On days 4 and 6, the medium is replaced. On day 8, cell number is determined with a Coulter Counter.

An increase in the number of HUVEC cells indicates that the polypeptide of the invention may proliferate vascular endothelial cells.

One skilled in the art could easily modify the exemplified studies to test the  
10 activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

#### EXAMPLE 39 – STIMULATORY EFFECT OF POLYPEPTIDES OF THE INVENTION ON THE PROLIFERATION OF VASCULAR 15 ENDOTHELIAL CELLS

For evaluation of mitogenic activity of tubulin tyrosine ligase proteins, the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium) assay with the electron coupling reagent PMS (phenazine methosulfate) was performed (CellTiter 96 AQ, Promega). Cells are  
20 seeded in a 96-well plate (5,000 cells/well) in 0.1 mL serum-supplemented medium and are allowed to attach overnight. After serum-starvation for 12 hours in 0.5% FBS, conditions (bFGF, VEGF165 or a polypeptide of the invention in 0.5% FBS) with or without Heparin (8 U/ml) are added to wells for 48 hours. 20 mg of MTS/PMS mixture (1:0.05) are added per well and allowed to incubate for 1 hour at 37°C before  
25 measuring the absorbance at 490 nm in an ELISA plate reader. Background absorbance from control wells (some media, no cells) is subtracted, and seven wells are performed in parallel for each condition. See, Leak et al. In Vitro Cell. Dev. Biol. 30A:512-518 (1994).

One skilled in the art could easily modify the exemplified studies to test the  
30 activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.



EXAMPLE 40 – INHIBITION OF PDGF-INDUCED VASCULAR SMOOTH  
MUSCLE CELL PROLIFERATION STIMULATORY EFFECT

HAoSMC proliferation can be measured, for example, by BrdUrd incorporation. Briefly, subconfluent, quiescent cells grown on the 4-chamber slides  
5 are transfected with CRP or FITC-labeled AT2-3LP. Then, the cells are pulsed with 10% calf serum and 6 mg/ml BrdUrd. After 24 h, immunocytochemistry is performed by using BrdUrd Staining Kit (Zymed Laboratories). In brief, the cells are incubated with the biotinylated mouse anti-BrdUrd antibody at 4 degrees C for 2 h after being exposed to denaturing solution and then incubated with the streptavidin-peroxidase  
10 and diaminobenzidine. After counterstaining with hematoxylin, the cells are mounted for microscopic examination, and the BrdUrd-positive cells are counted. The BrdUrd index is calculated as a percent of the BrdUrd-positive cells to the total cell number. In addition, the simultaneous detection of the BrdUrd staining (nucleus) and the FITC uptake (cytoplasm) is performed for individual cells by the concomitant use of bright  
15 field illumination and dark field-UV fluorescent illumination. See, Hayashida et al., J. Biol. Chem... 6:271(36):21985-21992 (1996).

One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

20

EXAMPLE 41 – STIMULATION OF ENDOTHELIAL MIGRATION

This example will be used to explore the possibility that a polypeptide of the invention may stimulate lymphatic endothelial cell migration.

Endothelial cell migration assays are performed using a 48 well  
25 microchemotaxis chamber (Neuroprobe Inc., Cabin John, MD; Falk, W., et al., J. Immunological Methods 1980;33:239-247). Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8 um (Nucleopore Corp. Cambridge, MA) are coated with 0.1% gelatin for at least 6 hours at room temperature and dried under sterile air. Test substances are diluted to appropriate concentrations in M199 supplemented with  
30 0.25% bovine serum albumin (BSA), and 25 ul of the final dilution is placed in the lower chamber of the modified Boyden apparatus. Subconfluent, early passage (2-6) HUVEC or BMEC cultures are washed and trypsinized for the minimum time

required to achieve cell detachment. After placing the filter between lower and upper chamber,  $2.5 \times 10^5$  cells suspended in 50 ul M199 containing 1% FBS are seeded in the upper compartment. The apparatus is then incubated for 5 hours at 37°C in a humidified chamber with 5% CO<sub>2</sub> to allow cell migration. After the incubation period, the filter is removed and the upper side of the filter with the non-migrated cells is scraped with a rubber policeman. The filters are fixed with methanol and stained with a Giemsa solution (Diff-Quick, Baxter, McGraw Park, IL). Migration is quantified by counting cells of three random high-power fields (40x) in each well, and all groups are performed in quadruplicate.

One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

#### EXAMPLE 42 – RAT CORNEAL WOUND HEALING MODEL

This animal model shows the effect of a polypeptide of the invention on neovascularization. The experimental protocol includes:

- a) Making a 1-1.5 mm long incision from the center of cornea into the stromal layer.
- b) Inserting a spatula below the lip of the incision facing the outer corner of the eye.
- c) Making a pocket (its base is 1-1.5 mm from the edge of the eye).
- d) Positioning a pellet, containing 50ng- 5ug of a polypeptide of the invention, within the pocket.
- e) Treatment with a polypeptide of the invention can also be applied topically to the corneal wounds in a dosage range of 20mg - 500mg (daily treatment for five days).

One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

EXAMPLE 43 – DIABETIC MOUSE AND GLUCOCORTICOID-IMPAIRED  
WOUND HEALING MODELS

A. Diabetic db+/db+ Mouse Model.

To demonstrate that a polypeptide of the invention accelerates the healing  
5 process, the genetically diabetic mouse model of wound healing is used. The full  
thickness wound healing model in the db+/db+ mouse is a well characterized,  
clinically relevant and reproducible model of impaired wound healing. Healing of the  
diabetic wound is dependent on formation of granulation tissue and re-  
epithelialization rather than contraction (Gartner, M.H. et al., J. Surg. Res. 52:389  
10 (1992); Greenhalgh, D.G. et al., Am. J. Pathol. 136:1235 (1990)).

The diabetic animals have many of the characteristic features observed in  
Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to  
their normal heterozygous (db+/-m) littermates. Mutant diabetic (db+/db+) mice have  
a single autosomal recessive mutation on chromosome 4 (db+) (Coleman et al. Proc.  
15 Natl. Acad. Sci. USA 77:283-293 (1982)). Animals show polyphagia, polydipsia and  
polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or  
normal insulin levels, and suppressed cell-mediated immunity (Mandel et al., J.  
Immunol. 120:1375 (1978); Debray-Sachs, M. et al., Clin. Exp. Immunol. 51(1):1-7  
(1983); Leiter et al., Am. J. of Pathol. 114:46-55 (1985)). Peripheral neuropathy,  
20 myocardial complications, and microvascular lesions, basement membrane thickening  
and glomerular filtration abnormalities have been described in these animals (Norido,  
F. et al., Exp. Neurol. 83(2):221-232 (1984); Robertson et al., Diabetes 29(1):60-67  
(1980); Giacomelli et al., Lab Invest. 40(4):460-473 (1979); Coleman, D.L., Diabetes  
31 (Suppl):1-6 (1982)). These homozygous diabetic mice develop hyperglycemia that  
25 is resistant to insulin analogous to human type II diabetes (Mandel et al., J. Immunol.  
120:1375-1377 (1978)).

The characteristics observed in these animals suggests that healing in this  
model may be similar to the healing observed in human diabetes (Greenhalgh, et al.,  
Am. J. of Pathol. 136:1235-1246 (1990)).

30 Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic  
(db+/-m) heterozygous littermates are used in this study (Jackson Laboratories). The  
animals are purchased at 6 weeks of age and are 8 weeks old at the beginning of the

study. Animals are individually housed and received food and water ad libitum. All manipulations are performed using aseptic techniques. The experiments are conducted according to the rules and guidelines of Bristol-Myers Squibb Company's Institutional Animal Care and Use Committee and the Guidelines for the Care and  
5 Use of Laboratory Animals.

Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., J. Exp. Med. 172:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized  
10 water. The dorsal region of the animal is shaved and the skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration of the  
15 experiment. Application of the treatment is given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily  
20 measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

A polypeptide of the invention is administered using at a range different doses,  
25 from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology and immunohistochemistry. Tissue specimens are placed in 10% neutral  
30 buffered formalin in tissue cassettes between biopsy sponges for further processing.

Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls) are evaluated: 1) Vehicle placebo control, 2) untreated group, and 3) treated group.

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total square area of the wound. Contraction is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm<sup>2</sup>, the corresponding size of the dermal punch. Calculations are made using the following formula:

$$[\text{Open area on day 8}] - [\text{Open area on day 1}] / [\text{Open area on day 1}]$$

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using a Reichert-Jung microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds are used to assess whether the healing process and the morphologic appearance of the repaired skin is altered by treatment with a polypeptide of the invention. This assessment included verification of the presence of cell accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and epidermal maturity (Greenhalgh, D.G. et al., Am. J. Pathol. 136:1235 (1990)). A calibrated lens micrometer is used by a blinded observer.

Tissue sections are also stained immunohistochemically with a polyclonal rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is used as a positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer can serve as a positive tissue control and human brain tissue can be used as a negative tissue control. Each specimen includes a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8, the lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation.

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

## B. Steroid Impaired Rat Model

The inhibition of wound healing by steroids has been well documented in various in vitro and in vivo systems (Wahl, Glucocorticoids and Wound healing. In: Anti-Inflammatory Steroid Action: Basic and Clinical Aspects. 280-302 (1989); Wahlet al., J. Immunol. 115: 476-481 (1975); Werb et al., J. Exp. Med. 147:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting angiogenesis, decreasing vascular permeability (Ebert et al., An. Intern. Med. 37:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck et al., Growth Factors. 5: 295-304 (1991); Haynes et al., J. Clin. Invest. 61: 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes et al., J. Clin. Invest. 61: 703-797 (1978); Wahl, "Glucocorticoids and wound healing", In: Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well establish phenomenon in rats (Beck et al., Growth Factors. 5: 295-304 (1991); Haynes et al., J. Clin. Invest. 61: 703-797 (1978); Wahl, "Glucocorticoids and wound healing", In: Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989); Pierce et al., Proc. Natl. Acad. Sci. USA 86: 2229-2233 (1989)).

To demonstrate that a polypeptide of the invention can accelerate the healing process, the effects of multiple topical applications of the polypeptide on full thickness excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

Young adult male Sprague Dawley rats weighing 250-300 g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and are 9 weeks old at the beginning of the study. The healing response of rats is impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water ad libitum. All manipulations are performed using aseptic techniques. This study would be conducted according to the rules and guidelines of Bristol-Myers Squibb Corporations Guidelines for the Care and Use of Laboratory Animals.

The wounding protocol is followed according to section A, above. On the day of wounding, animals are anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

The polypeptide of the invention is administered using at a range different doses, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

Four groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) are evaluated: 1) Untreated group 2) Vehicle placebo control 3) treated groups.

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total area of the wound. Closure is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm<sup>2</sup>, the corresponding size of the dermal punch. Calculations are made using the following formula:

$$[\text{Open area on day 8}] - [\text{Open area on day 1}] / [\text{Open area on day 1}]$$

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin is improved by treatment with a polypeptide of the invention. A calibrated lens micrometer is used by a blinded observer to determine the distance of the wound gap.

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

#### EXAMPLE 44 – SUPPRESSION OF TNF ALPHA-INDUCED ADHESION

##### MOLECULE EXPRESSION BY A POLYPEPTIDE OF THE INVENTION

The recruitment of lymphocytes to areas of inflammation and angiogenesis involves specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and tubulin tyrosine ligase protein participate in the modulation of the expression of these CAMs.

Tumor necrosis factor alpha (TNF-a), a potent proinflammatory cytokine, is a stimulator of all three CAMs on endothelial cells and may be involved in a wide variety of inflammatory responses, often resulting in a pathological outcome.

The potential of a polypeptide of the invention to mediate a suppression of TNF-a induced CAM expression can be examined. A modified ELISA assay which uses ECs as a solid phase absorbent is employed to measure the amount of CAM



expression on TNF- $\alpha$  treated ECs when co-stimulated with a member of the FGF family of proteins.

To perform the experiment, human umbilical vein endothelial cell (HUVEC) cultures are obtained from pooled cord harvests and maintained in growth medium (EGM-2; Clonetics, San Diego, CA) supplemented with 10% FCS and 1% penicillin/streptomycin in a 37 degree C humidified incubator containing 5% CO<sub>2</sub>. HUVECs are seeded in 96-well plates at concentrations of  $1 \times 10^4$  cells/well in EGM medium at 37 degree C for 18-24 hrs or until confluent. The monolayers are subsequently washed 3 times with a serum-free solution of RPMI-1640 supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, and treated with a given cytokine and/or tubulin tyrosine ligase protein(s) for 24 h at 37 degree C. Following incubation, the cells are then evaluated for CAM expression.

Human Umbilical Vein Endothelial cells (HUVECs) are grown in a standard 96 well plate to confluence. Growth medium is removed from the cells and replaced with 90  $\mu$ l of 199 Medium (10% FBS). Samples for testing and positive or negative controls are added to the plate in triplicate (in 10  $\mu$ l volumes). Plates are incubated at 37 degree C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100  $\mu$ l of 0.1% paraformaldehyde-PBS(with Ca<sup>++</sup> and Mg<sup>++</sup>) is added to each well. Plates are held at 4°C for 30 min.

Fixative is then removed from the wells and wells are washed 1X with PBS(+Ca,Mg)+0.5% BSA and drained. Do not allow the wells to dry. Add 10  $\mu$ l of diluted primary antibody to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10  $\mu$ g/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA.

Then add 20  $\mu$ l of diluted ExtrAvidin-Alkaline Phosphatase (1:5,000 dilution) to each well and incubated at 37°C for 30 min. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA. 1 tablet of p-Nitrophenol Phosphate pNPP is dissolved in 5 ml of glycine buffer (pH 10.4). 100  $\mu$ l of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphatase in glycine buffer: 1:5,000 (100) > 10-0.5 >

10-1 > 10-1.5. 5 µl of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100 µl of pNNP reagent must then be added to each of the standard wells. The plate must be incubated at 37°C for 4h. A volume of 50 µl of 3M NaOH is added to all wells. The results are  
5 quantified on a plate reader at 405 nm. The background subtraction option is used on blank wells filled with glycine buffer only. The template is set up to indicate the concentration of AP-conjugate in each standard well [ 5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

One skilled in the art could easily modify the exemplified studies to test the  
10 activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

#### EXAMPLE 45 – SITE DIRECTED/SITE-SPECIFIC MUTAGENESIS

*In vitro* site-directed mutagenesis is an invaluable technique for studying  
15 protein structure-function relationships and gene expression, for example, as well as for vector modification. Site-directed mutagenesis can also be used for creating any of one or more of the mutants of the present invention, particularly the conservative and/or non-conservative amino acid substitution mutants of the present invention. Approaches utilizing single stranded DNA (ssDNA) as the template have been  
20 reported (e.g., T.A. Kunkel et al., 1985, *Proc. Natl. Acad. Sci. USA*), 82:488-492; M.A. Vandeyar et al., 1988, *Gene*, 65(1):129-133; M. Sugimoto et al., 1989, *Anal. Biochem.*, 179(2):309-311; and J.W. Taylor et al., 1985, *Nuc. Acids. Res.*, 13(24):8765-8785).

The use of PCR in site-directed mutagenesis accomplishes strand separation  
25 by using a denaturing step to separate the complementary strands and to allow efficient polymerization of the PCR primers. PCR site-directed mutagenesis methods thus permit site specific mutations to be incorporated in virtually any double stranded plasmid, thus eliminating the need for re-subcloning into M13-based bacteriophage vectors or single-stranded rescue. (M.P. Weiner et al., 1995, *Molecular Biology: Current Innovations and Future Trends*, Eds. A.M. Griffin and H.G. Griffin, Horizon  
30 Scientific Press, Norfolk, UK; and C. Papworth et al., 1996, *Strategies*, 9(3):3-4).

A protocol for performing site-directed mutagenesis, particularly employing the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA; U.S. Patent Nos. 5,789,166 and 5,923,419) is provided for making point mutations, to switch or substitute amino acids, and to delete or insert single or multiple amino acids in the  
5 RATL1d6 amino acid sequence of this invention.

#### Primer Design

For primer design using this protocol, the mutagenic oligonucleotide primers are designed individually according to the desired mutation. The following considerations should be made for designing mutagenic primers: 1) Both of the  
10 mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid; 2) Primers should be between 25 and 45 bases in length, and the melting temperature ( $T_m$ ) of the primers should be greater than, or equal to, 78°C. The following formula is commonly used for estimating the  $T_m$  of  
15 primers:  $T = 81.5 + 0.41 (\%GC) - 675/N - \%mismatch$ . For calculating  $T_m$ ,  $N$  is the primer length in bases; and values for %GC and % mismatch are whole numbers. For calculating  $T_m$  for primers intended to introduce insertions or deletions, a modified version of the above formula is employed:  $T = 81.5 + 0.41 (\%GC) - 675/N$ , where  $N$   
20 does not include the bases which are being inserted or deleted; 3) The desired mutation (deletion or insertion) should be in the middle of the primer with approximately 10-15 bases of correct sequence on both sides; 4) The primers  
optimally should have a minimum GC content of 40%, and should terminate in one or more C or G bases; 5) Primers need not be 5'-phosphorylated, but must be purified either by fast polynucleotide liquid chromatography (FPLC) or by polyacrylamide gel  
25 electrophoresis (PAGE). Failure to purify the primers results in a significant decrease in mutation efficiency; and 6) It is important that primer concentration is in excess. It is suggested to vary the amount of template while keeping the concentration of the primers constantly in excess (QuikChange™ Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA).

#### Protocol for Setting Up the Reactions

30 Using the above-described primer design, two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleic acid sequence, are synthesized. The resulting oligonucleotide primers are purified.

A control reaction is prepared using 5 µl 10x reaction buffer (100mM KCl; 100mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 200mM Tris-HCl, pH 8.8; 20mM MgSO<sub>4</sub>; 1% Triton® X-100; 1 mg/ml nuclease-free bovine serum albumin, BSA); 2 µl (10ng) of pWhitescript™, 4.5-kb control plasmid (5 ng/µl); 1.25 µl (125 ng) of oligonucleotide control primer #1 (34-mer, 100 ng/µl); 1.25 µl (125 ng) of oligonucleotide control primer #2 (34-mer, 100 ng/µl); 1 µl of dNTP mix; double distilled H<sub>2</sub>O; to a final volume of 50 µl. Thereafter, 1 µl of DNA polymerase (*PfuTurbo*® DNA Polymerase, Stratagene), (2.5U/µl) is added. *PfuTurbo*® DNA Polymerase is stated to have 6-fold higher fidelity in DNA synthesis than does *Taq* polymerase. To maximize temperature cycling performance, use of thin-walled test tubes is suggested to ensure optimum contact with the heating blocks of the temperature cycler.

The sample reaction is prepared by combining 5 µl of 10x reaction buffer; x µl (5-50 ng) of dsDNA template; x µl (125 ng) of oligonucleotide primer #1; x µl (5-50 ng) of dsDNA template; x µl (125 ng) of oligonucleotide primer #2; 1 µl of dNTP mix; and ddH<sub>2</sub>O to a final volume of 50 µl. Thereafter, 1 µl of DNA polymerase (*PfuTurbo* DNA Polymerase, Stratagene), (2.5U/µl) is added.

It is suggested that if the thermal cycler does not have a hot-top assembly, each reaction should be overlaid with approximately 30 µl of mineral oil.

#### Cycling the Reactions

Each reaction is cycled using the following cycling parameters:

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12-18	95°C	30 seconds
		55°C	1 minute
		68°C	2 minutes/kb of plasmid length

For the control reaction, a 12-minute extension time is used and the reaction is run for 12 cycles. Segment 2 of the above cycling parameters is adjusted in accordance with the type of mutation desired. For example, for point mutations, 12 cycles are used; for single amino acid changes, 16 cycles are used; and for multiple amino acid deletions or insertions, 18 cycles are used. Following the temperature cycling, the reaction is placed on ice for 2 minutes to cool the reaction to ≤37°C.

### Digesting the Products and Transforming Competent Cells

One  $\mu\text{l}$  of the *DpnI* restriction enzyme (10U/ $\mu\text{l}$ ) is added directly (below mineral oil overlay) to each amplification reaction using a small, pointed pipette tip. The reaction mixture is gently and thoroughly mixed by pipetting the solution up and  
5 down several times. The reaction mixture is then centrifuged for 1 minute in a microcentrifuge. Immediately thereafter, each reaction is incubated at 37°C for 1 hour to digest the parental (i.e., the non-mutated) supercoiled dsDNA.

Competent cells (i.e., XL1-Blue supercompetent cells, Stratagene) are thawed gently on ice. For each control and sample reaction to be transformed, 50  $\mu\text{l}$  of the  
10 supercompetent cells are aliquotted to a prechilled test tube (Falcon 2059 polypropylene). Next, 1  $\mu\text{l}$  of the *DpnI*-digested DNA is transferred from the control and the sample reactions to separate aliquots of the supercompetent cells. The transformation reactions are gently swirled to mix and incubated for 30 minutes on ice. Thereafter, the transformation reactions are heat-pulsed for 45 seconds at 42°C  
15 for 2 minutes.

0.5 ml of NZY+ broth, preheated to 42°C is added to the transformation reactions which are then incubated at 37°C for 1 hour with shaking at 225-250 rpm. An aliquot of each transformation reaction is plated on agar plates containing the appropriate antibiotic for the vector. For the mutagenesis and transformation controls,  
20 cells are spread on LB-ampicillin agar plates containing 80  $\mu\text{g}/\text{ml}$  of X-gal and 20mM IPTG. Transformation plates are incubated for >16 hours at 37°C.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above  
25 teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other disclosures) in the Background of the Invention, Detailed Description, and Examples is hereby incorporated herein by reference. Further, the hard copy of the sequence  
30 listing submitted herewith and the corresponding computer readable form are both incorporated herein by reference in their entireties.

TABLE IV

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1	N	MET	1	39.078	12.834	68.219
2	CA	MET	1	38.470	12.062	67.126
3	C	MET	1	36.989	12.378	66.980
4	O	MET	1	36.371	13.008	67.846
5	CB	MET	1	38.644	10.569	67.365
6	CG	MET	1	40.112	10.173	67.285
7	SD	MET	1	40.891	10.457	65.678
8	CE	MET	1	42.551	9.850	66.054
9	N	ALA	2	36.440	11.938	65.865
10	CA	ALA	2	35.020	12.139	65.596
11	C	ALA	2	34.338	10.813	65.304
12	O	ALA	2	34.979	9.858	64.846
13	CB	ALA	2	34.865	13.066	64.397
14	N	SER	3	33.016	10.874	65.293
15	CA	SER	3	32.191	9.686	65.035
16	C	SER	3	32.242	9.282	63.575
17	O	SER	3	32.311	8.087	63.276
18	CB	SER	3	30.740	10.014	65.335
19	OG	SER	3	30.673	10.482	66.665
20	N	SER	4	32.526	10.255	62.724
21	CA	SER	4	32.633	9.995	61.288
22	C	SER	4	33.908	9.219	60.978
23	O	SER	4	33.868	8.247	60.214
24	CB	SER	4	32.699	11.337	60.571
25	OG	SER	4	31.574	12.111	60.966
26	N	ILE	5	34.914	9.433	61.811
27	CA	ILE	5	36.197	8.766	61.632
28	C	ILE	5	36.136	7.354	62.194
29	O	ILE	5	36.636	6.427	61.548
30	CB	ILE	5	37.252	9.576	62.373
31	CG1	ILE	5	37.267	11.013	61.865
32	CG2	ILE	5	38.629	8.942	62.223
33	CD1	ILE	5	38.319	11.845	62.589
34	N	LEU	6	35.275	7.156	63.178
35	CA	LEU	6	35.082	5.818	63.738
36	C	LEU	6	34.283	4.941	62.789
37	O	LEU	6	34.655	3.779	62.583
38	CB	LEU	6	34.309	5.930	65.043
39	CG	LEU	6	35.101	6.650	66.121
40	CD1	LEU	6	34.224	6.860	67.346
41	CD2	LEU	6	36.360	5.869	66.483
42	N	LYS	7	33.383	5.551	62.036
43	CA	LYS	7	32.587	4.785	61.079

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
44	C	LYS	7	33.396	4.442	59.834
45	O	LYS	7	33.296	3.309	59.345
46	CB	LYS	7	31.355	5.604	60.722
47	CG	LYS	7	30.587	5.949	61.992
48	CD	LYS	7	29.346	6.789	61.723
49	CE	LYS	7	28.706	7.236	63.033
50	NZ	LYS	7	28.427	6.084	63.905
51	N	TRP	8	34.371	5.274	59.508
52	CA	TRP	8	35.256	4.934	58.397
53	C	TRP	8	36.284	3.887	58.807
54	O	TRP	8	36.399	2.876	58.108
55	CB	TRP	8	35.954	6.189	57.881
56	CG	TRP	8	35.041	7.209	57.221
57	CD1	TRP	8	35.134	8.578	57.348
58	CD2	TRP	8	33.921	6.958	56.339
59	NE1	TRP	8	34.145	9.149	56.616
60	CE2	TRP	8	33.397	8.217	55.997
61	CE3	TRP	8	33.340	5.803	55.833
62	CZ2	TRP	8	32.297	8.303	55.156
63	CZ3	TRP	8	32.238	5.898	54.992
64	CH2	TRP	8	31.718	7.143	54.655
65	N	VAL	9	36.774	3.953	60.034
66	CA	VAL	9	37.766	2.968	60.478
67	C	VAL	9	37.152	1.586	60.692
68	O	VAL	9	37.691	0.615	60.143
69	CB	VAL	9	38.423	3.458	61.764
70	CG1	VAL	9	39.313	2.384	62.380
71	CG2	VAL	9	39.227	4.728	61.512
72	N	VAL	10	35.917	1.536	61.165
73	CA	VAL	10	35.270	0.236	61.362
74	C	VAL	10	34.711	-0.332	60.060
75	O	VAL	10	34.774	-1.553	59.866
76	CB	VAL	10	34.185	0.380	62.423
77	CG1	VAL	10	33.372	-0.901	62.581
78	CG2	VAL	10	34.814	0.770	63.755
79	N	SER	11	34.468	0.520	59.076
80	CA	SER	11	34.084	-0.009	57.766
81	C	SER	11	35.318	-0.470	56.990
82	O	SER	11	35.228	-1.482	56.287
83	CB	SER	11	33.296	1.030	56.971
84	OG	SER	11	34.130	2.147	56.702
85	N	HIS	12	36.488	0.042	57.345
86	CA	HIS	12	37.732	-0.467	56.753
87	C	HIS	12	38.077	-1.826	57.355
88	O	HIS	12	38.413	-2.754	56.610

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
89	CB	HIS	12	38.897	0.478	57.049
90	CG	HIS	12	38.796	1.896	56.518
91	ND1	HIS	12	38.139	2.321	55.421
92	CD2	HIS	12	39.385	3.003	57.082
93	CE1	HIS	12	38.292	3.655	55.299
94	NE2	HIS	12	39.063	4.076	56.326
95	N	GLN	13	37.720	-2.008	58.618
96	CA	GLN	13	37.949	-3.285	59.309
97	C	GLN	13	36.909	-4.347	58.948
98	O	GLN	13	37.148	-5.543	59.144
99	CB	GLN	13	37.887	-3.009	60.805
100	CG	GLN	13	38.962	-2.008	61.207
101	CD	GLN	13	38.749	-1.545	62.643
102	OE1	GLN	13	37.666	-1.073	63.012
103	NE2	GLN	13	39.809	-1.632	63.426
104	N	SER	14	35.810	-3.915	58.351
105	CA	SER	14	34.799	-4.835	57.829
106	C	SER	14	34.975	-5.089	56.330
107	O	SER	14	34.138	-5.774	55.727
108	CB	SER	14	33.426	-4.223	58.077
109	OG	SER	14	33.303	-3.993	59.474
110	N	CYS	15	36.022	-4.512	55.751
111	CA	CYS	15	36.316	-4.600	54.311
112	C	CYS	15	35.152	-4.065	53.486
113	O	CYS	15	34.659	-4.715	52.556
114	CB	CYS	15	36.628	-6.044	53.934
115	SG	CYS	15	38.049	-6.774	54.782
116	N	SER	16	34.716	-2.876	53.856
117	CA	SER	16	33.572	-2.246	53.207
118	C	SER	16	34.021	-1.179	52.223
119	O	SER	16	34.968	-0.421	52.475
120	CB	SER	16	32.705	-1.616	54.286
121	OG	SER	16	32.480	-2.608	55.280
122	N	ARG	17	33.299	-1.119	51.117
123	CA	ARG	17	33.542	-0.124	50.065
124	C	ARG	17	32.975	1.234	50.481
125	O	ARG	17	31.809	1.556	50.227
126	CB	ARG	17	32.855	-0.634	48.802
127	CG	ARG	17	33.214	0.174	47.563
128	CD	ARG	17	34.726	0.239	47.369
129	NE	ARG	17	35.075	0.700	46.015
130	CZ	ARG	17	35.197	1.977	45.647
131	NH1	ARG	17	34.965	2.959	46.521
132	NH2	ARG	17	35.535	2.271	44.390
133	N	SER	18	33.810	1.994	51.166



Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
134	CA	SER	18	33.403	3.281	51.725
135	C	SER	18	33.545	4.400	50.697
136	O	SER	18	34.314	4.289	49.734
137	CB	SER	18	34.265	3.546	52.951
138	OG	SER	18	34.133	2.435	53.828
139	N	SER	19	32.731	5.427	50.868
140	CA	SER	19	32.714	6.545	49.920
141	C	SER	19	32.575	7.899	50.612
142	O	SER	19	31.554	8.202	51.241
143	CB	SER	19	31.557	6.333	48.952
144	OG	SER	19	31.900	5.247	48.104
145	N	ARG	20	33.584	8.729	50.420
146	CA	ARG	20	33.608	10.075	50.999
147	C	ARG	20	33.009	11.095	50.027
148	O	ARG	20	33.477	11.239	48.890
149	CB	ARG	20	35.065	10.429	51.278
150	CG	ARG	20	35.194	11.786	51.957
151	CD	ARG	20	36.647	12.232	52.043
152	NE	ARG	20	37.439	11.318	52.875
153	CZ	ARG	20	38.676	10.934	52.556
154	NH1	ARG	20	39.422	10.300	53.461
155	NH2	ARG	20	39.226	11.346	51.412
156	N	SER	21	31.966	11.781	50.464
157	CA	SER	21	31.340	12.804	49.616
158	C	SER	21	30.904	14.041	50.391
159	O	SER	21	30.315	13.952	51.474
160	CB	SER	21	30.114	12.207	48.945
161	OG	SER	21	30.554	11.152	48.109
162	N	LYS	22	31.179	15.190	49.802
163	CA	LYS	22	30.704	16.465	50.346
164	C	LYS	22	29.283	16.724	49.850
165	O	LYS	22	28.872	16.161	48.826
166	CB	LYS	22	31.649	17.569	49.875
167	CG	LYS	22	33.053	17.324	50.403
168	CD	LYS	22	34.020	18.431	50.009
169	CE	LYS	22	35.443	18.079	50.425
170	NZ	LYS	22	35.505	17.761	51.860
171	N	PRO	23	28.554	17.609	50.519
172	CA	PRO	23	27.174	17.917	50.103
173	C	PRO	23	27.064	18.624	48.743
174	O	PRO	23	26.054	18.441	48.054
175	CB	PRO	23	26.624	18.786	51.193
176	CG	PRO	23	27.722	19.094	52.200
177	CD	PRO	23	28.952	18.337	51.730
178	N	ARG	24	28.176	19.152	48.246

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
179	CA	ARG	24	28.234	19.767	46.913
180	C	ARG	24	28.338	18.724	45.793
181	O	ARG	24	28.387	19.076	44.612
182	CB	ARG	24	29.481	20.635	46.862
183	CG	ARG	24	29.573	21.559	48.068
184	CD	ARG	24	30.889	22.325	48.055
185	NE	ARG	24	32.025	21.390	47.987
186	CZ	ARG	24	32.997	21.492	47.078
187	NH1	ARG	24	32.979	22.488	46.189
188	NH2	ARG	24	33.991	20.601	47.060
189	N	ASP	25	28.422	17.457	46.165
190	CA	ASP	25	28.442	16.369	45.194
191	C	ASP	25	27.046	15.789	44.953
192	O	ASP	25	26.918	14.869	44.135
193	CB	ASP	25	29.359	15.274	45.728
194	CG	ASP	25	30.804	15.762	45.821
195	OD1	ASP	25	31.394	15.968	44.770
196	OD2	ASP	25	31.239	16.071	46.924
197	N	GLN	26	26.042	16.306	45.652
198	CA	GLN	26	24.656	15.815	45.535
199	C	GLN	26	23.864	16.469	44.406
200	O	GLN	26	23.219	17.506	44.602
201	CB	GLN	26	23.923	16.103	46.844
202	CG	GLN	26	24.223	15.085	47.939
203	CD	GLN	26	23.460	13.786	47.682
204	OE1	GLN	26	23.497	13.232	46.579
205	NE2	GLN	26	22.710	13.362	48.684
206	N	ARG	27	23.817	15.794	43.272
207	CA	ARG	27	23.057	16.302	42.122
208	C	ARG	27	21.842	15.428	41.814
209	O	ARG	27	21.977	14.263	41.425
210	CB	ARG	27	23.982	16.358	40.910
211	CG	ARG	27	25.120	17.349	41.132
212	CD	ARG	27	24.581	18.754	41.386
213	NE	ARG	27	25.669	19.715	41.625
214	CZ	ARG	27	25.812	20.397	42.764
215	NH1	ARG	27	24.984	20.171	43.787
216	NH2	ARG	27	26.808	21.275	42.896
217	N	GLU	28	20.676	16.053	41.788
218	CA	GLU	28	19.411	15.327	41.561
219	C	GLU	28	19.011	15.280	40.073
220	O	GLU	28	17.844	15.485	39.709
221	CB	GLU	28	18.337	16.009	42.408
222	CG	GLU	28	17.065	15.177	42.544
223	CD	GLU	28	16.105	15.851	43.515

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
224	OE1	GLU	28	14.935	15.958	43.178
225	OE2	GLU	28	16.595	16.399	44.495
226	N	GLU	29	19.968	14.922	39.231
227	CA	GLU	29	19.759	14.891	37.777
228	C	GLU	29	18.682	13.876	37.403
229	O	GLU	29	18.685	12.752	37.917
230	CB	GLU	29	21.091	14.525	37.120
231	CG	GLU	29	21.030	14.506	35.592
232	CD	GLU	29	20.665	15.884	35.042
233	OE1	GLU	29	19.475	16.139	34.907
234	OE2	GLU	29	21.573	16.674	34.836
235	N	ALA	30	17.696	14.346	36.649
236	CA	ALA	30	16.564	13.532	36.181
237	C	ALA	30	15.659	13.028	37.301
238	O	ALA	30	15.054	11.957	37.167
239	CB	ALA	30	17.115	12.334	35.412
240	N	GLY	31	15.614	13.758	38.407
241	CA	GLY	31	14.840	13.334	39.579
242	C	GLY	31	15.489	12.151	40.302
243	O	GLY	31	14.817	11.423	41.041
244	N	SER	32	16.780	11.965	40.086
245	CA	SER	32	17.482	10.818	40.651
246	C	SER	32	18.499	11.271	41.683
247	O	SER	32	18.342	12.316	42.327
248	CB	SER	32	18.190	10.093	39.514
249	OG	SER	32	17.243	9.931	38.466
250	N	SER	33	19.536	10.473	41.844
251	CA	SER	33	20.551	10.790	42.848
252	C	SER	33	21.959	10.495	42.361
253	O	SER	33	22.422	9.350	42.409
254	CB	SER	33	20.260	9.955	44.078
255	OG	SER	33	18.997	10.357	44.588
256	N	ASP	34	22.636	11.541	41.925
257	CA	ASP	34	24.016	11.441	41.446
258	C	ASP	34	25.000	12.006	42.471
259	O	ASP	34	24.887	13.164	42.886
260	CB	ASP	34	24.094	12.232	40.146
261	CG	ASP	34	25.532	12.319	39.662
262	OD1	ASP	34	26.125	13.377	39.830
263	OD2	ASP	34	26.017	11.321	39.154
264	N	LEU	35	25.974	11.193	42.843
265	CA	LEU	35	26.960	11.586	43.852
266	C	LEU	35	28.399	11.391	43.376
267	O	LEU	35	28.816	10.279	43.022
268	CB	LEU	35	26.734	10.701	45.070

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
269	CG	LEU	35	27.533	11.144	46.287
270	CD1	LEU	35	27.072	12.518	46.759
271	CD2	LEU	35	27.390	10.128	47.414
272	N	SER	36	29.138	12.486	43.347
273	CA	SER	36	30.589	12.390	43.150
274	C	SER	36	31.261	12.039	44.477
275	O	SER	36	31.071	12.719	45.494
276	CB	SER	36	31.130	13.705	42.610
277	OG	SER	36	32.542	13.576	42.511
278	N	SER	37	32.021	10.960	44.455
279	CA	SER	37	32.612	10.433	45.686
280	C	SER	37	34.055	9.959	45.543
281	O	SER	37	34.398	9.189	44.639
282	CB	SER	37	31.768	9.252	46.135
283	OG	SER	37	32.368	8.750	47.318
284	N	ARG	38	34.863	10.359	46.507
285	CA	ARG	38	36.249	9.898	46.613
286	C	ARG	38	36.301	8.685	47.530
287	O	ARG	38	35.324	8.388	48.225
288	CB	ARG	38	37.060	11.012	47.258
289	CG	ARG	38	36.938	12.298	46.461
290	CD	ARG	38	37.407	13.498	47.269
291	NE	ARG	38	36.580	13.639	48.477
292	CZ	ARG	38	35.468	14.375	48.538
293	NH1	ARG	38	35.086	15.112	47.491
294	NH2	ARG	38	34.768	14.423	49.670
295	N	GLN	39	37.409	7.970	47.522
296	CA	GLN	39	37.578	6.920	48.533
297	C	GLN	39	38.085	7.511	49.843
298	O	GLN	39	37.845	8.689	50.133
299	CB	GLN	39	38.493	5.825	48.021
300	CG	GLN	39	37.817	5.104	46.865
301	CD	GLN	39	38.717	4.002	46.331
302	OE1	GLN	39	39.355	3.272	47.098
303	NE2	GLN	39	38.813	3.945	45.015
304	N	ASP	40	38.625	6.660	50.699
305	CA	ASP	40	39.007	7.110	52.048
306	C	ASP	40	40.167	6.340	52.681
307	O	ASP	40	40.484	5.207	52.297
308	CB	ASP	40	37.781	7.049	52.962
309	CG	ASP	40	36.841	5.903	52.585
310	OD1	ASP	40	37.255	4.755	52.672
311	OD2	ASP	40	35.759	6.212	52.109
312	N	ALA	41	40.801	6.996	53.643
313	CA	ALA	41	41.893	6.393	54.421

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
314	C	ALA	41	41.740	6.662	55.915
315	O	ALA	41	40.646	6.967	56.405
316	CB	ALA	41	43.219	6.985	53.972
317	N	GLU	42	42.842	6.458	56.624
318	CA	GLU	42	42.927	6.760	58.062
319	C	GLU	42	44.388	6.751	58.528
320	O	GLU	42	45.095	5.741	58.412
321	CB	GLU	42	42.045	5.794	58.868
322	CG	GLU	42	42.429	4.316	58.779
323	CD	GLU	42	43.360	3.937	59.931
324	OE1	GLU	42	43.883	2.832	59.902
325	OE2	GLU	42	43.411	4.707	60.881
326	N	ASN	43	44.821	7.891	59.040
327	CA	ASN	43	46.199	8.065	59.522
328	C	ASN	43	46.312	9.423	60.212
329	O	ASN	43	45.824	10.421	59.671
330	CB	ASN	43	47.145	8.003	58.321
331	CG	ASN	43	48.604	7.960	58.761
332	OD1	ASN	43	49.218	8.998	59.037
333	ND2	ASN	43	49.107	6.749	58.910
334	N	ALA	44	47.107	9.507	61.267
335	CA	ALA	44	47.211	10.764	62.026
336	C	ALA	44	47.996	11.873	61.311
337	O	ALA	44	47.811	13.053	61.626
338	CB	ALA	44	47.877	10.463	63.364
339	N	GLU	45	48.782	11.521	60.305
340	CA	GLU	45	49.489	12.534	59.519
341	C	GLU	45	48.848	12.699	58.141
342	O	GLU	45	49.131	13.675	57.436
343	CB	GLU	45	50.939	12.099	59.328
344	CG	GLU	45	51.633	11.754	60.643
345	CD	GLU	45	51.627	12.939	61.605
346	OE1	GLU	45	51.043	12.779	62.667
347	OE2	GLU	45	52.388	13.866	61.364
348	N	ALA	46	47.960	11.780	57.793
349	CA	ALA	46	47.347	11.775	56.459
350	C	ALA	46	46.127	10.861	56.405
351	O	ALA	46	46.177	9.761	55.841
352	CB	ALA	46	48.376	11.284	55.446
353	N	LYS	47	45.013	11.356	56.915
354	CA	LYS	47	43.781	10.560	56.930
355	C	LYS	47	42.944	10.736	55.666
356	O	LYS	47	42.140	9.856	55.333
357	CB	LYS	47	42.965	10.995	58.145
358	CG	LYS	47	42.756	12.506	58.156

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
359	CD	LYS	47	41.926	12.968	59.347
360	CE	LYS	47	41.698	14.475	59.291
361	NZ	LYS	47	41.019	14.853	58.040
362	N	LEU	48	43.325	11.703	54.851
363	CA	LEU	48	42.454	12.154	53.771
364	C	LEU	48	42.959	11.742	52.389
365	O	LEU	48	43.160	12.608	51.531
366	CB	LEU	48	42.400	13.676	53.861
367	CG	LEU	48	41.119	14.258	53.275
368	CD1	LEU	48	39.928	13.917	54.164
369	CD2	LEU	48	41.237	15.771	53.134
370	N	ARG	49	43.196	10.459	52.167
371	CA	ARG	49	43.600	10.083	50.808
372	C	ARG	49	42.360	9.767	49.973
373	O	ARG	49	41.514	8.931	50.317
374	CB	ARG	49	44.627	8.944	50.780
375	CG	ARG	49	44.026	7.545	50.715
376	CD	ARG	49	45.096	6.482	50.503
377	NE	ARG	49	46.057	6.463	51.613
378	CZ	ARG	49	46.686	5.351	52.001
379	NH1	ARG	49	47.524	5.387	53.040
380	NH2	ARG	49	46.455	4.199	51.367
381	N	GLY	50	42.233	10.520	48.898
382	CA	GLY	50	41.113	10.335	47.979
383	C	GLY	50	41.516	9.472	46.792
384	O	GLY	50	41.877	9.988	45.728
385	N	LEU	51	41.534	8.167	47.012
386	CA	LEU	51	41.749	7.233	45.902
387	C	LEU	51	40.598	7.453	44.931
388	O	LEU	51	39.440	7.421	45.368
389	CB	LEU	51	41.756	5.807	46.439
390	CG	LEU	51	42.889	5.584	47.432
391	CD1	LEU	51	42.738	4.243	48.142
392	CD2	LEU	51	44.249	5.690	46.749
393	N	PRO	52	40.889	7.465	43.639
394	CA	PRO	52	40.410	8.550	42.775
395	C	PRO	52	38.978	8.996	43.052
396	O	PRO	52	38.718	9.683	44.046
397	CB	PRO	52	40.645	8.022	41.397
398	CG	PRO	52	41.767	6.993	41.504
399	CD	PRO	52	42.052	6.855	42.997
400	N	GLY	53	38.049	8.613	42.200
401	CA	GLY	53	36.675	9.042	42.453
402	C	GLY	53	35.674	8.456	41.478
403	O	GLY	53	36.017	8.112	40.340

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
404	N	GLN	54	34.440	8.381	41.941
405	CA	GLN	54	33.316	7.910	41.129
406	C	GLN	54	32.239	8.989	41.002
407	O	GLN	54	32.203	9.952	41.775
408	CB	GLN	54	32.726	6.647	41.761
409	CG	GLN	54	32.194	6.877	43.176
410	CD	GLN	54	33.063	6.172	44.221
411	OE1	GLN	54	32.914	6.393	45.427
412	NE2	GLN	54	33.947	5.314	43.743
413	N	LEU	55	31.462	8.877	39.940
414	CA	LEU	55	30.305	9.750	39.698
415	C	LEU	55	29.108	8.819	39.549
416	O	LEU	55	28.641	8.513	38.446
417	CB	LEU	55	30.585	10.552	38.425
418	CG	LEU	55	29.682	11.761	38.196
419	CD1	LEU	55	28.487	11.460	37.298
420	CD2	LEU	55	29.285	12.441	39.499
421	N	VAL	56	28.684	8.307	40.688
422	CA	VAL	56	27.761	7.174	40.701
423	C	VAL	56	26.363	7.590	41.150
424	O	VAL	56	26.200	8.588	41.858
425	CB	VAL	56	28.374	6.150	41.665
426	CG1	VAL	56	28.336	6.644	43.108
427	CG2	VAL	56	27.745	4.765	41.560
428	N	ASP	57	25.347	6.938	40.611
429	CA	ASP	57	24.032	7.074	41.232
430	C	ASP	57	24.159	6.400	42.594
431	O	ASP	57	24.645	5.264	42.684
432	CB	ASP	57	22.946	6.420	40.377
433	CG	ASP	57	21.560	6.694	40.969
434	OD1	ASP	57	20.898	7.613	40.502
435	OD2	ASP	57	21.200	5.991	41.906
436	N	ILE	58	23.660	7.046	43.634
437	CA	ILE	58	23.952	6.592	44.998
438	C	ILE	58	23.234	5.309	45.410
439	O	ILE	58	23.733	4.626	46.309
440	CB	ILE	58	23.587	7.686	45.982
441	CG1	ILE	58	23.708	9.059	45.348
442	CG2	ILE	58	24.518	7.600	47.181
443	CD1	ILE	58	23.341	10.146	46.349
444	N	ALA	59	22.284	4.847	44.612
445	CA	ALA	59	21.641	3.550	44.855
446	C	ALA	59	22.492	2.350	44.413
447	O	ALA	59	22.049	1.204	44.539
448	CB	ALA	59	20.318	3.526	44.108

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
449	N	CYS	60	23.670	2.606	43.861
450	CA	CYS	60	24.619	1.526	43.587
451	C	CYS	60	25.473	1.270	44.829
452	O	CYS	60	26.083	0.204	44.976
453	CB	CYS	60	25.502	1.930	42.414
454	SG	CYS	60	24.622	2.309	40.880
455	N	LYS	61	25.492	2.249	45.718
456	CA	LYS	61	26.057	2.061	47.053
457	C	LYS	61	24.922	1.578	47.948
458	O	LYS	61	23.780	2.024	47.799
459	CB	LYS	61	26.606	3.390	47.565
460	CG	LYS	61	27.683	3.960	46.645
461	CD	LYS	61	28.931	3.080	46.600
462	CE	LYS	61	29.988	3.666	45.669
463	NZ	LYS	61	31.195	2.824	45.630
464	N	VAL	62	25.211	0.648	48.839
465	CA	VAL	62	24.127	0.081	49.649
466	C	VAL	62	23.948	0.745	51.014
467	O	VAL	62	22.877	0.591	51.613
468	CB	VAL	62	24.363	-1.417	49.815
469	CG1	VAL	62	24.165	-2.146	48.491
470	CG2	VAL	62	25.742	-1.717	50.395
471	N	CYS	63	24.901	1.560	51.436
472	CA	CYS	63	24.817	2.190	52.761
473	C	CYS	63	25.467	3.569	52.783
474	O	CYS	63	26.656	3.715	52.475
475	CB	CYS	63	25.518	1.312	53.796
476	SG	CYS	63	24.752	-0.279	54.184
477	N	GLN	64	24.686	4.562	53.171
478	CA	GLN	64	25.230	5.914	53.361
479	C	GLN	64	24.906	6.453	54.755
480	O	GLN	64	23.745	6.459	55.179
481	CB	GLN	64	24.640	6.835	52.300
482	CG	GLN	64	25.112	8.278	52.449
483	CD	GLN	64	24.352	9.165	51.472
484	OE1	GLN	64	24.129	10.356	51.728
485	NE2	GLN	64	23.935	8.560	50.373
486	N	ALA	65	25.945	6.808	55.492
487	CA	ALA	65	25.761	7.484	56.782
488	C	ALA	65	25.819	8.990	56.565
489	O	ALA	65	26.784	9.483	55.969
490	CB	ALA	65	26.874	7.057	57.733
491	N	TYR	66	24.808	9.718	57.007
492	CA	TYR	66	24.844	11.149	56.704
493	C	TYR	66	25.304	11.994	57.882



Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
494	O	TYR	66	24.684	12.066	58.949
495	CB	TYR	66	23.526	11.637	56.130
496	CG	TYR	66	23.778	12.381	54.821
497	CD1	TYR	66	25.076	12.433	54.327
498	CD2	TYR	66	22.744	12.996	54.128
499	CE1	TYR	66	25.345	13.099	53.141
500	CE2	TYR	66	23.012	13.662	52.938
501	CZ	TYR	66	24.311	13.711	52.447
502	OH	TYR	66	24.578	14.356	51.262
503	N	LEU	67	26.378	12.709	57.594
504	CA	LEU	67	27.087	13.531	58.576
505	C	LEU	67	26.753	15.028	58.492
506	O	LEU	67	27.508	15.851	59.023
507	CB	LEU	67	28.576	13.293	58.359
508	CG	LEU	67	28.891	11.797	58.407
509	CD1	LEU	67	30.283	11.499	57.862
510	CD2	LEU	67	28.701	11.212	59.805
511	N	GLY	68	25.665	15.376	57.819
512	CA	GLY	68	25.215	16.778	57.764
513	C	GLY	68	24.657	17.184	59.127
514	O	GLY	68	23.633	16.658	59.576
515	N	GLN	69	25.341	18.107	59.780
516	CA	GLN	69	25.053	18.370	61.189
517	C	GLN	69	24.542	19.784	61.464
518	O	GLN	69	23.330	20.032	61.450
519	CB	GLN	69	26.352	18.135	61.941
520	CG	GLN	69	26.106	17.830	63.408
521	CD	GLN	69	27.430	17.880	64.145
522	OE1	GLN	69	28.178	18.860	64.035
523	NE2	GLN	69	27.722	16.804	64.847
524	N	LEU	70	25.462	20.685	61.770
525	CA	LEU	70	25.087	22.042	62.186
526	C	LEU	70	24.370	22.801	61.076
527	O	LEU	70	24.544	22.524	59.883
528	CB	LEU	70	26.307	22.840	62.659
529	CG	LEU	70	27.267	23.276	61.549
530	CD1	LEU	70	27.876	24.637	61.868
531	CD2	LEU	70	28.362	22.249	61.263
532	N	GLU	71	23.515	23.710	61.518
533	CA	GLU	71	22.706	24.564	60.638
534	C	GLU	71	21.847	23.755	59.671
535	O	GLU	71	22.150	23.690	58.472
536	CB	GLU	71	23.615	25.501	59.845
537	CG	GLU	71	24.398	26.440	60.754
538	CD	GLU	71	25.278	27.376	59.926

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
539	OE1	GLU	71	26.407	26.988	59.657
540	OE2	GLU	71	24.884	28.523	59.771
541	N	HIS	72	20.751	23.214	60.180
542	CA	HIS	72	19.785	22.493	59.335
543	C	HIS	72	18.870	23.462	58.587
544	O	HIS	72	17.757	23.775	59.021
545	CB	HIS	72	18.940	21.566	60.202
546	CG	HIS	72	19.404	20.121	60.254
547	ND1	HIS	72	20.671	19.659	60.247
548	CD2	HIS	72	18.581	19.021	60.322
549	CE1	HIS	72	20.653	18.311	60.296
550	NE2	HIS	72	19.361	17.917	60.344
551	N	GLU	73	19.388	23.967	57.481
552	CA	GLU	73	18.662	24.923	56.644
553	C	GLU	73	17.986	24.224	55.472
554	O	GLU	73	18.089	23.000	55.316
555	CB	GLU	73	19.673	25.940	56.134
556	CG	GLU	73	20.388	26.612	57.301
557	CD	GLU	73	21.512	27.505	56.789
558	OE1	GLU	73	21.420	27.921	55.643
559	OE2	GLU	73	22.465	27.696	57.530
560	N	ASP	74	17.485	25.026	54.545
561	CA	ASP	74	16.783	24.486	53.371
562	C	ASP	74	17.731	23.831	52.364
563	O	ASP	74	17.342	22.849	51.727
564	CB	ASP	74	16.025	25.618	52.684
565	CG	ASP	74	14.966	26.197	53.619
566	OD1	ASP	74	14.917	27.414	53.727
567	OD2	ASP	74	14.335	25.414	54.316
568	N	ILE	75	19.012	24.159	52.447
569	CA	ILE	75	20.014	23.505	51.596
570	C	ILE	75	20.349	22.103	52.117
571	O	ILE	75	20.453	21.157	51.325
572	CB	ILE	75	21.270	24.372	51.610
573	CG1	ILE	75	20.974	25.781	51.104
574	CG2	ILE	75	22.378	23.735	50.778
575	CD1	ILE	75	20.554	25.779	49.637
576	N	ASP	76	20.161	21.924	53.417
577	CA	ASP	76	20.404	20.631	54.058
578	C	ASP	76	19.207	19.723	53.808
579	O	ASP	76	19.383	18.581	53.361
580	CB	ASP	76	20.578	20.888	55.554
581	CG	ASP	76	20.760	19.596	56.343
582	OD1	ASP	76	21.890	19.127	56.391
583	OD2	ASP	76	19.816	19.213	57.015

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
584	N	THR	77	18.057	20.368	53.692
585	CA	THR	77	16.810	19.664	53.394
586	C	THR	77	16.740	19.245	51.924
587	O	THR	77	16.287	18.130	51.640
588	CB	THR	77	15.658	20.612	53.713
589	OG1	THR	77	15.792	21.041	55.063
590	CG2	THR	77	14.300	19.938	53.552
591	N	SER	78	17.448	19.963	51.066
592	CA	SER	78	17.501	19.606	49.644
593	C	SER	78	18.419	18.410	49.401
594	O	SER	78	18.024	17.483	48.682
595	CB	SER	78	18.015	20.806	48.858
596	OG	SER	78	17.109	21.882	49.059
597	N	ALA	79	19.461	18.289	50.210
598	CA	ALA	79	20.344	17.120	50.109
599	C	ALA	79	19.726	15.893	50.781
600	O	ALA	79	19.965	14.757	50.350
601	CB	ALA	79	21.676	17.452	50.770
602	N	ASP	80	18.770	16.138	51.664
603	CA	ASP	80	18.015	15.054	52.293
604	C	ASP	80	16.867	14.583	51.391
605	O	ASP	80	16.505	13.401	51.424
606	CB	ASP	80	17.452	15.559	53.619
607	CG	ASP	80	18.540	15.989	54.599
608	OD1	ASP	80	19.623	15.420	54.557
609	OD2	ASP	80	18.262	16.889	55.383
610	N	ALA	81	16.480	15.417	50.437
611	CA	ALA	81	15.473	15.018	49.451
612	C	ALA	81	16.101	14.172	48.346
613	O	ALA	81	15.470	13.220	47.868
614	CB	ALA	81	14.841	16.270	48.854
615	N	VAL	82	17.403	14.332	48.161
616	CA	VAL	82	18.137	13.442	47.254
617	C	VAL	82	18.408	12.098	47.937
618	O	VAL	82	18.281	11.046	47.296
619	CB	VAL	82	19.452	14.109	46.864
620	CG1	VAL	82	20.189	13.289	45.813
621	CG2	VAL	82	19.214	15.520	46.341
622	N	GLU	83	18.426	12.123	49.264
623	CA	GLU	83	18.535	10.892	50.057
624	C	GLU	83	17.240	10.093	50.045
625	O	GLU	83	17.288	8.859	49.971
626	CB	GLU	83	18.859	11.256	51.500
627	CG	GLU	83	20.276	11.783	51.628
628	CD	GLU	83	21.227	10.691	51.166

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
629	OE1	GLU	83	21.234	9.658	51.821
630	OE2	GLU	83	21.696	10.801	50.038
631	N	ASP	84	16.136	10.777	49.801
632	CA	ASP	84	14.848	10.102	49.673
633	C	ASP	84	14.780	9.289	48.384
634	O	ASP	84	14.348	8.129	48.426
635	CB	ASP	84	13.769	11.174	49.640
636	CG	ASP	84	12.384	10.546	49.619
637	OD1	ASP	84	11.468	11.207	49.149
638	OD2	ASP	84	12.245	9.466	50.174
639	N	LEU	85	15.487	9.747	47.363
640	CA	LEU	85	15.479	9.038	46.085
641	C	LEU	85	16.428	7.845	46.100
642	O	LEU	85	16.073	6.794	45.554
643	CB	LEU	85	15.896	10.022	45.005
644	CG	LEU	85	14.951	11.215	44.972
645	CD1	LEU	85	15.555	12.372	44.193
646	CD2	LEU	85	13.583	10.830	44.419
647	N	THR	86	17.462	7.902	46.927
648	CA	THR	86	18.355	6.745	47.039
649	C	THR	86	17.735	5.678	47.935
650	O	THR	86	17.804	4.488	47.599
651	CB	THR	86	19.697	7.171	47.628
652	OG1	THR	86	19.597	7.326	49.033
653	CG2	THR	86	20.194	8.482	47.059
654	N	GLU	87	16.883	6.108	48.853
655	CA	GLU	87	16.237	5.172	49.767
656	C	GLU	87	15.080	4.460	49.072
657	O	GLU	87	14.964	3.235	49.200
658	CB	GLU	87	15.742	5.970	50.968
659	CG	GLU	87	15.376	5.081	52.149
660	CD	GLU	87	15.050	5.949	53.363
661	OE1	GLU	87	13.933	5.852	53.852
662	OE2	GLU	87	15.944	6.651	53.823
663	N	ALA	88	14.474	5.143	48.111
664	CA	ALA	88	13.410	4.536	47.300
665	C	ALA	88	13.956	3.640	46.186
666	O	ALA	88	13.228	2.800	45.647
667	CB	ALA	88	12.573	5.652	46.688
668	N	GLU	89	15.248	3.750	45.917
669	CA	GLU	89	15.908	2.851	44.967
670	C	GLU	89	16.651	1.713	45.674
671	O	GLU	89	17.522	1.092	45.052
672	CB	GLU	89	16.878	3.640	44.098
673	CG	GLU	89	16.191	4.738	43.292

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
674	CD	GLU	89	15.078	4.173	42.414
675	OE1	GLU	89	13.932	4.499	42.692
676	OE2	GLU	89	15.406	3.615	41.376
677	N	TRP	90	16.412	1.572	46.973
678	CA	TRP	90	16.915	0.463	47.808
679	C	TRP	90	18.333	0.676	48.332
680	O	TRP	90	19.130	-0.268	48.389
681	CB	TRP	90	16.827	-0.881	47.083
682	CG	TRP	90	15.427	-1.450	46.948
683	CD1	TRP	90	14.525	-1.219	45.932
684	CD2	TRP	90	14.785	-2.359	47.868
685	NE1	TRP	90	13.395	-1.926	46.191
686	CE2	TRP	90	13.508	-2.623	47.338
687	CE3	TRP	90	15.186	-2.950	49.056
688	CZ2	TRP	90	12.649	-3.479	48.011
689	CZ3	TRP	90	14.319	-3.807	49.723
690	CH2	TRP	90	13.056	-4.070	49.202
691	N	GLU	91	18.637	1.896	48.740
692	CA	GLU	91	19.885	2.130	49.476
693	C	GLU	91	19.569	2.457	50.933
694	O	GLU	91	18.858	3.429	51.217
695	CB	GLU	91	20.676	3.279	48.855
696	CG	GLU	91	21.949	3.525	49.660
697	CD	GLU	91	22.763	4.691	49.116
698	OE1	GLU	91	22.141	5.602	48.584
699	OE2	GLU	91	23.907	4.806	49.540
700	N	ASP	92	20.112	1.661	51.842
701	CA	ASP	92	19.925	1.912	53.275
702	C	ASP	92	20.742	3.133	53.695
703	O	ASP	92	21.900	3.314	53.295
704	CB	ASP	92	20.371	0.675	54.046
705	CG	ASP	92	19.944	0.757	55.508
706	OD1	ASP	92	18.920	1.376	55.763
707	OD2	ASP	92	20.649	0.209	56.345
708	N	LEU	93	20.081	4.032	54.402
709	CA	LEU	93	20.722	5.288	54.802
710	C	LEU	93	20.498	5.582	56.278
711	O	LEU	93	19.358	5.824	56.699
712	CB	LEU	93	20.074	6.433	54.046
713	CG	LEU	93	19.938	6.228	52.549
714	CD1	LEU	93	18.942	7.227	51.995
715	CD2	LEU	93	21.274	6.346	51.841
716	N	THR	94	21.589	5.807	56.986
717	CA	THR	94	21.501	6.115	58.420
718	C	THR	94	21.434	7.629	58.636

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
719	O	THR	94	22.449	8.326	58.763
720	CB	THR	94	22.671	5.484	59.170
721	OG1	THR	94	23.887	5.935	58.599
722	CG2	THR	94	22.639	3.965	59.054
723	N	GLN	95	20.212	8.117	58.488
724	CA	GLN	95	19.888	9.536	58.646
725	C	GLN	95	18.390	9.757	58.850
726	O	GLN	95	18.018	10.683	59.578
727	CB	GLN	95	20.398	10.317	57.423
728	CG	GLN	95	20.200	9.628	56.065
729	CD	GLN	95	18.840	9.895	55.419
730	OE1	GLN	95	18.474	11.042	55.144
731	NE2	GLN	95	18.113	8.823	55.146
732	N	GLN	96	17.620	8.737	58.498
733	CA	GLN	96	16.165	8.849	58.278
734	C	GLN	96	15.707	10.274	57.957
735	O	GLN	96	15.362	11.048	58.858
736	CB	GLN	96	15.402	8.319	59.478
737	CG	GLN	96	14.458	7.181	59.086
738	CD	GLN	96	13.484	7.605	57.987
739	OE1	GLN	96	13.045	8.760	57.922
740	NE2	GLN	96	13.235	6.681	57.076
741	N	TYR	97	15.444	10.482	56.678
742	CA	TYR	97	15.101	11.800	56.131
743	C	TYR	97	13.760	12.337	56.623
744	O	TYR	97	13.700	13.484	57.088
745	CB	TYR	97	15.051	11.626	54.616
746	CG	TYR	97	14.305	12.705	53.838
747	CD1	TYR	97	14.563	14.052	54.060
748	CD2	TYR	97	13.350	12.329	52.902
749	CE1	TYR	97	13.881	15.022	53.336
750	CE2	TYR	97	12.668	13.296	52.179
751	CZ	TYR	97	12.940	14.639	52.393
752	OH	TYR	97	12.343	15.594	51.602
753	N	TYR	98	12.791	11.453	56.791
754	CA	TYR	98	11.466	11.905	57.201
755	C	TYR	98	11.449	12.142	58.700
756	O	TYR	98	10.900	13.153	59.143
757	CB	TYR	98	10.430	10.850	56.832
758	CG	TYR	98	10.248	10.639	55.332
759	CD1	TYR	98	10.717	9.481	54.724
760	CD2	TYR	98	9.599	11.608	54.576
761	CE1	TYR	98	10.543	9.294	53.359
762	CE2	TYR	98	9.424	11.422	53.212
763	CZ	TYR	98	9.895	10.265	52.608

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
764	OH	TYR	98	9.683	10.065	51.261
765	N	SER	99	12.348	11.463	59.388
766	CA	SER	99	12.456	11.632	60.831
767	C	SER	99	13.194	12.919	61.176
768	O	SER	99	12.724	13.664	62.045
769	CB	SER	99	13.221	10.442	61.373
770	OG	SER	99	12.546	9.274	60.929
771	N	LEU	100	14.131	13.313	60.328
772	CA	LEU	100	14.833	14.588	60.534
773	C	LEU	100	13.898	15.772	60.326
774	O	LEU	100	13.779	16.632	61.210
775	CB	LEU	100	15.959	14.717	59.514
776	CG	LEU	100	17.032	13.652	59.676
777	CD1	LEU	100	18.076	13.774	58.571
778	CD2	LEU	100	17.687	13.735	61.050
779	N	VAL	101	13.084	15.679	59.287
780	CA	VAL	101	12.186	16.780	58.927
781	C	VAL	101	10.908	16.814	59.772
782	O	VAL	101	10.275	17.870	59.893
783	CB	VAL	101	11.849	16.601	57.446
784	CG1	VAL	101	10.853	17.638	56.937
785	CG2	VAL	101	13.118	16.639	56.603
786	N	HIS	102	10.577	15.716	60.431
787	CA	HIS	102	9.362	15.708	61.251
788	C	HIS	102	9.651	15.792	62.749
789	O	HIS	102	8.717	15.982	63.535
790	CB	HIS	102	8.548	14.448	60.959
791	CG	HIS	102	8.044	14.309	59.530
792	ND1	HIS	102	7.800	15.301	58.650
793	CD2	HIS	102	7.744	13.129	58.891
794	CE1	HIS	102	7.372	14.772	57.486
795	NE2	HIS	102	7.338	13.429	57.636
796	N	GLY	103	10.910	15.689	63.143
797	CA	GLY	103	11.230	15.758	64.573
798	C	GLY	103	12.706	16.022	64.867
799	O	GLY	103	13.338	15.284	65.632
800	N	ASP	104	13.216	17.121	64.338
801	CA	ASP	104	14.588	17.563	64.646
802	C	ASP	104	14.754	17.927	66.126
803	O	ASP	104	13.760	18.018	66.857
804	CB	ASP	104	14.933	18.773	63.776
805	CG	ASP	104	13.954	19.921	64.008
806	OD1	ASP	104	14.035	20.531	65.071
807	OD2	ASP	104	13.055	20.072	63.195
808	N	ALA	105	15.984	18.270	66.490

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
809	CA	ALA	105	16.405	18.626	67.865
810	C	ALA	105	15.317	19.139	68.807
811	O	ALA	105	14.684	18.328	69.496
812	CB	ALA	105	17.516	19.664	67.766
813	N	PHE	106	14.964	20.410	68.703
814	CA	PHE	106	14.001	20.990	69.656
815	C	PHE	106	12.520	20.746	69.319
816	O	PHE	106	11.660	20.937	70.187
817	CB	PHE	106	14.262	22.492	69.751
818	CG	PHE	106	15.607	22.861	70.375
819	CD1	PHE	106	16.583	23.494	69.615
820	CD2	PHE	106	15.850	22.572	71.712
821	CE1	PHE	106	17.803	23.829	70.190
822	CE2	PHE	106	17.070	22.906	72.285
823	CZ	PHE	106	18.047	23.534	71.525
824	N	ILE	107	12.237	20.178	68.157
825	CA	ILE	107	10.843	19.964	67.745
826	C	ILE	107	10.362	18.541	68.050
827	O	ILE	107	9.158	18.323	68.254
828	CB	ILE	107	10.747	20.291	66.256
829	CG1	ILE	107	11.068	21.766	66.035
830	CG2	ILE	107	9.374	19.958	65.681
831	CD1	ILE	107	10.889	22.173	64.577
832	N	SER	108	11.309	17.679	68.391
833	CA	SER	108	11.012	16.271	68.711
834	C	SER	108	10.370	16.073	70.086
835	O	SER	108	9.768	15.022	70.357
836	CB	SER	108	12.324	15.507	68.685
837	OG	SER	108	13.150	16.038	69.714
838	N	ASN	109	10.307	17.142	70.860
839	CA	ASN	109	9.642	17.091	72.153
840	C	ASN	109	8.141	17.325	72.090
841	O	ASN	109	7.448	17.029	73.069
842	CB	ASN	109	10.322	18.063	73.088
843	CG	ASN	109	11.292	17.196	73.858
844	OD1	ASN	109	12.337	16.794	73.328
845	ND2	ASN	109	10.741	16.643	74.917
846	N	SER	110	7.622	17.521	70.888
847	CA	SER	110	6.168	17.612	70.710
848	C	SER	110	5.468	16.252	70.864
849	O	SER	110	4.245	16.214	71.030
850	CB	SER	110	5.881	18.178	69.322
851	OG	SER	110	6.396	17.270	68.355
852	N	ARG	111	6.228	15.165	70.879
853	CA	ARG	111	5.659	13.846	71.186



Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
854	C	ARG	111	5.987	13.407	72.619
855	O	ARG	111	5.313	12.538	73.182
856	CB	ARG	111	6.297	12.830	70.249
857	CG	ARG	111	6.351	13.311	68.806
858	CD	ARG	111	7.092	12.292	67.950
859	NE	ARG	111	8.407	11.989	68.541
860	CZ	ARG	111	8.973	10.781	68.489
861	NH1	ARG	111	8.359	9.781	67.852
862	NH2	ARG	111	10.161	10.575	69.062
863	N	ASN	112	6.915	14.115	73.242
864	CA	ASN	112	7.539	13.660	74.494
865	C	ASN	112	7.248	14.606	75.662
866	O	ASN	112	8.041	14.685	76.610
867	CB	ASN	112	9.056	13.580	74.285
868	CG	ASN	112	9.527	12.309	73.559
869	OD1	ASN	112	10.046	11.384	74.203
870	ND2	ASN	112	9.513	12.347	72.236
871	N	TYR	113	6.033	15.129	75.696
872	CA	TYR	113	5.677	16.187	76.656
873	C	TYR	113	5.409	15.729	78.095
874	O	TYR	113	5.533	16.554	79.005
875	CB	TYR	113	4.434	16.897	76.113
876	CG	TYR	113	3.776	17.888	77.076
877	CD1	TYR	113	4.379	19.112	77.343
878	CD2	TYR	113	2.576	17.556	77.695
879	CE1	TYR	113	3.784	20.001	78.230
880	CE2	TYR	113	1.980	18.444	78.582
881	CZ	TYR	113	2.588	19.663	78.848
882	OH	TYR	113	2.008	20.536	79.745
883	N	PHE	114	5.309	14.433	78.338
884	CA	PHE	114	4.803	13.977	79.641
885	C	PHE	114	5.804	13.981	80.803
886	O	PHE	114	5.367	13.788	81.943
887	CB	PHE	114	4.239	12.573	79.478
888	CG	PHE	114	3.091	12.459	78.481
889	CD1	PHE	114	1.904	13.143	78.707
890	CD2	PHE	114	3.231	11.666	77.350
891	CE1	PHE	114	0.858	13.035	77.800
892	CE2	PHE	114	2.186	11.557	76.443
893	CZ	PHE	114	0.999	12.242	76.668
894	N	SER	115	7.092	14.174	80.561
895	CA	SER	115	8.011	14.301	81.697
896	C	SER	115	9.059	15.372	81.457
897	O	SER	115	9.664	15.830	82.431
898	CB	SER	115	8.720	12.988	82.012

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
899	OG	SER	115	9.791	12.794	81.098
900	N	GLN	116	9.231	15.794	80.215
901	CA	GLN	116	10.215	16.833	79.883
902	C	GLN	116	9.997	18.117	80.674
903	O	GLN	116	8.913	18.358	81.221
904	CB	GLN	116	10.051	17.186	78.415
905	CG	GLN	116	8.647	17.726	78.172
906	CD	GLN	116	8.566	18.415	76.820
907	OE1	GLN	116	8.245	17.792	75.801
908	NE2	GLN	116	8.957	19.675	76.810
909	N	CYS	117	11.042	18.923	80.754
910	CA	CYS	117	10.891	20.261	81.327
911	C	CYS	117	9.927	21.044	80.447
912	O	CYS	117	9.909	20.843	79.226
913	CB	CYS	117	12.244	20.962	81.337
914	SG	CYS	117	13.552	20.152	82.284
915	N	GLN	118	9.069	21.843	81.061
916	CA	GLN	118	8.123	22.655	80.282
917	C	GLN	118	8.891	23.513	79.289
918	O	GLN	118	9.947	24.060	79.623
919	CB	GLN	118	7.320	23.557	81.209
920	CG	GLN	118	6.526	22.767	82.241
921	CD	GLN	118	5.740	23.741	83.113
922	OE1	GLN	118	5.430	24.857	82.685
923	NE2	GLN	118	5.442	23.316	84.328
924	N	ALA	119	8.421	23.516	78.054
925	CA	ALA	119	9.127	24.214	76.977
926	C	ALA	119	8.211	25.173	76.236
927	O	ALA	119	7.415	24.758	75.385
928	CB	ALA	119	9.658	23.178	75.996
929	N	LEU	120	8.383	26.454	76.502
930	CA	LEU	120	7.563	27.460	75.825
931	C	LEU	120	8.139	27.820	74.457
932	O	LEU	120	9.136	28.546	74.344
933	CB	LEU	120	7.462	28.703	76.701
934	CG	LEU	120	6.628	29.797	76.040
935	CD1	LEU	120	5.192	29.334	75.816
936	CD2	LEU	120	6.654	31.072	76.872
937	N	LEU	121	7.565	27.189	73.448
938	CA	LEU	121	7.826	27.543	72.052
939	C	LEU	121	7.051	28.824	71.754
940	O	LEU	121	5.823	28.856	71.911
941	CB	LEU	121	7.337	26.370	71.193
942	CG	LEU	121	7.667	26.452	69.699
943	CD1	LEU	121	6.698	27.315	68.895

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
944	CD2	LEU	121	9.118	26.837	69.444
945	N	ASN	122	7.766	29.890	71.433
946	CA	ASN	122	7.081	31.158	71.148
947	C	ASN	122	7.918	32.131	70.319
948	O	ASN	122	9.107	31.913	70.062
949	CB	ASN	122	6.696	31.816	72.469
950	CG	ASN	122	5.259	32.316	72.364
951	OD1	ASN	122	5.020	33.441	71.906
952	ND2	ASN	122	4.342	31.377	72.497
953	N	ARG	123	7.232	33.143	69.812
954	CA	ARG	123	7.870	34.242	69.082
955	C	ARG	123	7.602	35.564	69.792
956	O	ARG	123	8.383	36.518	69.692
957	CB	ARG	123	7.248	34.358	67.697
958	CG	ARG	123	7.446	33.131	66.822
959	CD	ARG	123	6.789	33.362	65.468
960	NE	ARG	123	7.067	32.263	64.535
961	CZ	ARG	123	6.774	32.330	63.235
962	NH1	ARG	123	6.178	33.418	62.742
963	NH2	ARG	123	7.065	31.306	62.431
964	N	ILE	124	6.498	35.591	70.520
965	CA	ILE	124	6.016	36.823	71.145
966	C	ILE	124	6.770	37.096	72.436
967	O	ILE	124	6.674	36.322	73.398
968	CB	ILE	124	4.529	36.633	71.432
969	CG1	ILE	124	3.809	36.155	70.177
970	CG2	ILE	124	3.888	37.919	71.942
971	CD1	ILE	124	2.333	35.892	70.452
972	N	THR	125	7.309	38.301	72.525
973	CA	THR	125	8.147	38.698	73.664
974	C	THR	125	7.392	38.810	74.997
975	O	THR	125	7.977	38.463	76.030
976	CB	THR	125	8.787	40.038	73.304
977	OG1	THR	125	9.638	39.824	72.184
978	CG2	THR	125	9.637	40.611	74.434
979	N	SER	126	6.077	38.963	74.943
980	CA	SER	126	5.279	39.131	76.164
981	C	SER	126	4.724	37.828	76.747
982	O	SER	126	4.107	37.876	77.818
983	CB	SER	126	4.102	40.047	75.859
984	OG	SER	126	3.211	39.335	75.011
985	N	VAL	127	4.931	36.690	76.099
986	CA	VAL	127	4.397	35.441	76.664
987	C	VAL	127	5.414	34.816	77.613
988	O	VAL	127	5.080	34.385	78.729

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
989	CB	VAL	127	4.087	34.480	75.522
990	CG1	VAL	127	3.589	33.140	76.051
991	CG2	VAL	127	3.065	35.085	74.568
992	N	ASN	128	6.661	35.091	77.287
993	CA	ASN	128	7.806	34.602	78.057
994	C	ASN	128	7.881	35.094	79.511
995	O	ASN	128	8.038	34.212	80.362
996	CB	ASN	128	9.061	35.005	77.304
997	CG	ASN	128	8.864	34.685	75.828
998	OD1	ASN	128	8.507	33.559	75.450
999	ND2	ASN	128	9.110	35.687	75.007
1000	N	PRO	129	7.679	36.368	79.859
1001	CA	PRO	129	7.760	36.730	81.282
1002	C	PRO	129	6.658	36.144	82.176
1003	O	PRO	129	6.950	35.921	83.354
1004	CB	PRO	129	7.714	38.226	81.330
1005	CG	PRO	129	7.469	38.770	79.937
1006	CD	PRO	129	7.447	37.561	79.025
1007	N	GLN	130	5.564	35.646	81.618
1008	CA	GLN	130	4.513	35.056	82.455
1009	C	GLN	130	4.969	33.690	82.958
1010	O	GLN	130	5.108	33.479	84.172
1011	CB	GLN	130	3.271	34.880	81.592
1012	CG	GLN	130	2.915	36.176	80.874
1013	CD	GLN	130	1.683	35.964	80.001
1014	OE1	GLN	130	0.784	35.188	80.346
1015	NE2	GLN	130	1.674	36.628	78.859
1016	N	THR	131	5.545	32.945	82.030
1017	CA	THR	131	6.089	31.624	82.356
1018	C	THR	131	7.450	31.728	83.049
1019	O	THR	131	7.764	30.890	83.902
1020	CB	THR	131	6.207	30.818	81.068
1021	OG1	THR	131	7.074	31.510	80.180
1022	CG2	THR	131	4.852	30.671	80.386
1023	N	ASP	132	8.089	32.879	82.913
1024	CA	ASP	132	9.334	33.157	83.627
1025	C	ASP	132	9.069	33.515	85.089
1026	O	ASP	132	9.860	33.112	85.945
1027	CB	ASP	132	10.029	34.317	82.923
1028	CG	ASP	132	11.370	34.646	83.571
1029	OD1	ASP	132	11.770	35.798	83.474
1030	OD2	ASP	132	12.001	33.733	84.084
1031	N	ILE	133	7.873	33.988	85.404
1032	CA	ILE	133	7.507	34.216	86.807
1033	C	ILE	133	7.051	32.912	87.460

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1034	O	ILE	133	7.287	32.698	88.654
1035	CB	ILE	133	6.387	35.253	86.859
1036	CG1	ILE	133	6.881	36.603	86.356
1037	CG2	ILE	133	5.824	35.397	88.269
1038	CD1	ILE	133	5.758	37.633	86.344
1039	N	ASP	134	6.660	31.962	86.622
1040	CA	ASP	134	6.284	30.626	87.097
1041	C	ASP	134	7.503	29.723	87.309
1042	O	ASP	134	7.388	28.681	87.965
1043	CB	ASP	134	5.371	29.982	86.060
1044	CG	ASP	134	4.118	30.825	85.836
1045	OD1	ASP	134	3.637	30.832	84.710
1046	OD2	ASP	134	3.631	31.397	86.801
1047	N	GLY	135	8.653	30.134	86.800
1048	CA	GLY	135	9.895	29.400	87.050
1049	C	GLY	135	10.707	30.112	88.128
1050	O	GLY	135	11.212	29.479	89.065
1051	N	LEU	136	10.722	31.432	88.025
1052	CA	LEU	136	11.439	32.339	88.933
1053	C	LEU	136	12.843	31.827	89.247
1054	O	LEU	136	13.536	31.389	88.318
1055	CB	LEU	136	10.605	32.539	90.195
1056	CG	LEU	136	10.635	33.996	90.657
1057	CD1	LEU	136	10.238	34.940	89.527
1058	CD2	LEU	136	9.746	34.204	91.877
1059	N	ARG	137	13.177	31.763	90.532
1060	CA	ARG	137	14.527	31.448	91.056
1061	C	ARG	137	15.627	31.420	90.005
1062	O	ARG	137	15.952	32.453	89.418
1063	CB	ARG	137	14.473	30.112	91.781
1064	CG	ARG	137	13.800	30.287	93.135
1065	CD	ARG	137	14.574	31.310	93.957
1066	NE	ARG	137	13.944	31.555	95.261
1067	CZ	ARG	137	14.640	31.956	96.326
1068	NH1	ARG	137	14.019	32.180	97.486
1069	NH2	ARG	137	15.958	32.141	96.227
1070	N	ASN	138	16.197	30.258	89.764
1071	CA	ASN	138	17.203	30.133	88.703
1072	C	ASN	138	16.634	29.416	87.477
1073	O	ASN	138	17.366	29.112	86.527
1074	CB	ASN	138	18.396	29.365	89.262
1075	CG	ASN	138	19.003	30.121	90.443
1076	OD1	ASN	138	19.492	31.245	90.288
1077	ND2	ASN	138	18.918	29.524	91.621
1078	N	ILE	139	15.319	29.293	87.445

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1079	CA	ILE	139	14.649	28.317	86.584
1080	C	ILE	139	14.132	28.887	85.254
1081	O	ILE	139	12.918	28.879	85.006
1082	CB	ILE	139	13.504	27.745	87.424
1083	CG1	ILE	139	14.012	27.363	88.808
1084	CG2	ILE	139	12.860	26.521	86.788
1085	CD1	ILE	139	12.910	26.741	89.658
1086	N	TRP	140	15.035	29.307	84.377
1087	CA	TRP	140	14.600	29.663	83.015
1088	C	TRP	140	15.715	29.548	81.969
1089	O	TRP	140	16.650	30.357	81.924
1090	CB	TRP	140	14.028	31.074	83.019
1091	CG	TRP	140	12.980	31.302	81.947
1092	CD1	TRP	140	12.913	32.358	81.065
1093	CD2	TRP	140	11.853	30.447	81.648
1094	NE1	TRP	140	11.818	32.195	80.280
1095	CE2	TRP	140	11.153	31.070	80.601
1096	CE3	TRP	140	11.392	29.251	82.181
1097	CZ2	TRP	140	9.992	30.491	80.112
1098	CZ3	TRP	140	10.235	28.673	81.675
1099	CH2	TRP	140	9.536	29.292	80.645
1100	N	ILE	141	15.559	28.580	81.081
1101	CA	ILE	141	16.554	28.325	80.027
1102	C	ILE	141	16.111	28.871	78.672
1103	O	ILE	141	15.254	28.297	77.990
1104	CB	ILE	141	16.779	26.820	79.921
1105	CG1	ILE	141	17.311	26.279	81.237
1106	CG2	ILE	141	17.751	26.496	78.793
1107	CD1	ILE	141	18.635	26.942	81.591
1108	N	ILE	142	16.799	29.915	78.250
1109	CA	ILE	142	16.498	30.617	77.002
1110	C	ILE	142	17.324	30.112	75.813
1111	O	ILE	142	18.535	30.344	75.749
1112	CB	ILE	142	16.824	32.077	77.282
1113	CG1	ILE	142	15.925	32.643	78.371
1114	CG2	ILE	142	16.736	32.928	76.034
1115	CD1	ILE	142	16.210	34.125	78.585
1116	N	LYS	143	16.682	29.407	74.895
1117	CA	LYS	143	17.388	28.946	73.687
1118	C	LYS	143	16.501	28.997	72.437
1119	O	LYS	143	15.273	29.029	72.548
1120	CB	LYS	143	17.917	27.542	73.964
1121	CG	LYS	143	16.866	26.630	74.579
1122	CD	LYS	143	17.489	25.331	75.080
1123	CE	LYS	143	16.460	24.456	75.789

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1124	NZ	LYS	143	17.088	23.245	76.340
1125	N	PRO	144	17.109	29.162	71.271
1126	CA	PRO	144	16.354	29.160	70.008
1127	C	PRO	144	15.836	27.765	69.663
1128	O	PRO	144	16.376	26.762	70.140
1129	CB	PRO	144	17.323	29.641	68.973
1130	CG	PRO	144	18.711	29.714	69.588
1131	CD	PRO	144	18.544	29.340	71.051
1132	N	ALA	145	14.816	27.709	68.820
1133	CA	ALA	145	14.230	26.423	68.407
1134	C	ALA	145	14.811	25.848	67.112
1135	O	ALA	145	14.335	24.808	66.641
1136	CB	ALA	145	12.732	26.613	68.215
1137	N	ALA	146	15.827	26.496	66.564
1138	CA	ALA	146	16.372	26.120	65.250
1139	C	ALA	146	17.078	24.765	65.254
1140	O	ALA	146	16.443	23.718	65.084
1141	CB	ALA	146	17.345	27.205	64.803
1142	N	LYS	147	18.395	24.801	65.319
1143	CA	LYS	147	19.152	23.551	65.355
1144	C	LYS	147	19.660	23.324	66.773
1145	O	LYS	147	18.990	22.655	67.568
1146	CB	LYS	147	20.286	23.608	64.324
1147	CG	LYS	147	21.099	22.312	64.241
1148	CD	LYS	147	20.217	21.084	64.052
1149	CE	LYS	147	21.048	19.809	64.121
1150	NZ	LYS	147	20.191	18.615	64.052
1151	N	SER	148	20.790	23.944	67.079
1152	CA	SER	148	21.483	23.768	68.359
1153	C	SER	148	22.855	24.430	68.300
1154	O	SER	148	22.985	25.636	68.047
1155	CB	SER	148	21.643	22.274	68.634
1156	OG	SER	148	22.028	21.613	67.434
1157	N	ARG	149	23.839	23.647	68.714
1158	CA	ARG	149	25.266	23.935	68.506
1159	C	ARG	149	25.727	25.182	69.250
1160	O	ARG	149	26.452	26.009	68.686
1161	CB	ARG	149	25.538	24.105	67.008
1162	CG	ARG	149	24.967	22.957	66.174
1163	CD	ARG	149	25.618	21.610	66.469
1164	NE	ARG	149	24.760	20.516	65.985
1165	CZ	ARG	149	24.787	19.289	66.510
1166	NH1	ARG	149	25.757	18.955	67.363
1167	NH2	ARG	149	23.934	18.357	66.079
1168	N	GLY	150	25.254	25.338	70.476

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1169	CA	GLY	150	25.677	26.449	71.330
1170	C	GLY	150	25.073	27.822	71.069
1171	O	GLY	150	25.307	28.731	71.881
1172	N	ARG	151	24.322	27.982	69.990
1173	CA	ARG	151	23.797	29.302	69.635
1174	C	ARG	151	22.797	29.820	70.651
1175	O	ARG	151	21.683	29.309	70.793
1176	CB	ARG	151	23.168	29.262	68.249
1177	CG	ARG	151	24.189	29.657	67.188
1178	CD	ARG	151	23.565	29.711	65.797
1179	NE	ARG	151	24.439	30.427	64.850
1180	CZ	ARG	151	25.244	29.833	63.965
1181	NH1	ARG	151	25.327	28.501	63.922
1182	NH2	ARG	151	25.988	30.573	63.140
1183	N	ASP	152	23.267	30.823	71.377
1184	CA	ASP	152	22.486	31.557	72.373
1185	C	ASP	152	21.735	30.637	73.335
1186	O	ASP	152	20.509	30.521	73.262
1187	CB	ASP	152	21.508	32.459	71.627
1188	CG	ASP	152	22.246	33.420	70.700
1189	OD1	ASP	152	22.794	34.388	71.206
1190	OD2	ASP	152	22.332	33.115	69.518
1191	N	ILE	153	22.478	29.900	74.143
1192	CA	ILE	153	21.840	29.100	75.196
1193	C	ILE	153	22.045	29.831	76.522
1194	O	ILE	153	23.082	29.687	77.183
1195	CB	ILE	153	22.465	27.708	75.258
1196	CG1	ILE	153	22.566	27.039	73.885
1197	CG2	ILE	153	21.679	26.819	76.217
1198	CD1	ILE	153	21.213	26.654	73.301
1199	N	VAL	154	21.076	30.662	76.862
1200	CA	VAL	154	21.191	31.562	78.015
1201	C	VAL	154	20.399	31.077	79.229
1202	O	VAL	154	19.165	31.142	79.258
1203	CB	VAL	154	20.664	32.931	77.587
1204	CG1	VAL	154	20.786	33.956	78.710
1205	CG2	VAL	154	21.376	33.429	76.334
1206	N	CYS	155	21.110	30.589	80.229
1207	CA	CYS	155	20.464	30.262	81.506
1208	C	CYS	155	20.258	31.532	82.328
1209	O	CYS	155	21.237	32.160	82.745
1210	CB	CYS	155	21.363	29.307	82.283
1211	SG	CYS	155	20.809	28.917	83.959
1212	N	MET	156	19.012	31.917	82.544
1213	CA	MET	156	18.772	33.138	83.314



Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1214	C	MET	156	18.043	32.869	84.628
1215	O	MET	156	17.166	32.003	84.756
1216	CB	MET	156	18.037	34.169	82.451
1217	CG	MET	156	16.564	33.865	82.186
1218	SD	MET	156	15.384	34.456	83.426
1219	CE	MET	156	15.826	36.208	83.431
1220	N	ASP	157	18.506	33.585	85.633
1221	CA	ASP	157	17.864	33.585	86.940
1222	C	ASP	157	17.211	34.930	87.246
1223	O	ASP	157	17.248	35.885	86.456
1224	CB	ASP	157	18.880	33.220	88.021
1225	CG	ASP	157	20.169	34.035	87.948
1226	OD1	ASP	157	20.168	35.159	88.431
1227	OD2	ASP	157	21.156	33.484	87.479
1228	N	ARG	158	16.562	34.941	88.396
1229	CA	ARG	158	15.856	36.094	88.961
1230	C	ARG	158	16.670	37.384	88.936
1231	O	ARG	158	17.900	37.380	88.816
1232	CB	ARG	158	15.494	35.721	90.394
1233	CG	ARG	158	16.726	35.230	91.148
1234	CD	ARG	158	16.358	34.543	92.458
1235	NE	ARG	158	15.669	35.456	93.380
1236	CZ	ARG	158	16.139	35.738	94.597
1237	NH1	ARG	158	17.296	35.210	95.003
1238	NH2	ARG	158	15.466	36.567	95.398
1239	N	VAL	159	15.913	38.473	88.900
1240	CA	VAL	159	16.377	39.878	88.809
1241	C	VAL	159	17.389	40.172	87.691
1242	O	VAL	159	18.208	41.092	87.813
1243	CB	VAL	159	16.883	40.389	90.168
1244	CG1	VAL	159	15.803	40.242	91.235
1245	CG2	VAL	159	18.188	39.759	90.653
1246	N	GLU	160	17.284	39.462	86.579
1247	CA	GLU	160	18.090	39.813	85.414
1248	C	GLU	160	17.270	40.638	84.437
1249	O	GLU	160	16.225	40.208	83.935
1250	CB	GLU	160	18.617	38.562	84.734
1251	CG	GLU	160	19.640	37.847	85.604
1252	CD	GLU	160	20.199	36.671	84.818
1253	OE1	GLU	160	20.171	36.752	83.599
1254	OE2	GLU	160	20.493	35.654	85.431
1255	N	GLU	161	17.858	41.756	84.049
1256	CA	GLU	161	17.188	42.710	83.159
1257	C	GLU	161	17.306	42.317	81.686
1258	O	GLU	161	16.519	42.784	80.853

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1259	CB	GLU	161	17.829	44.070	83.394
1260	CG	GLU	161	17.657	44.500	84.847
1261	CD	GLU	161	18.509	45.733	85.129
1262	OE1	GLU	161	18.088	46.551	85.933
1263	OE2	GLU	161	19.598	45.795	84.576
1264	N	ILE	162	18.102	41.291	81.425
1265	CA	ILE	162	18.313	40.803	80.063
1266	C	ILE	162	17.230	39.837	79.580
1267	O	ILE	162	17.331	39.370	78.441
1268	CB	ILE	162	19.672	40.119	79.982
1269	CG1	ILE	162	19.734	38.898	80.892
1270	CG2	ILE	162	20.781	41.102	80.336
1271	CD1	ILE	162	21.072	38.181	80.753
1272	N	LEU	163	16.157	39.651	80.337
1273	CA	LEU	163	15.071	38.771	79.889
1274	C	LEU	163	14.397	39.364	78.653
1275	O	LEU	163	14.324	38.679	77.625
1276	CB	LEU	163	14.070	38.617	81.039
1277	CG	LEU	163	12.989	37.552	80.810
1278	CD1	LEU	163	11.768	38.052	80.040
1279	CD2	LEU	163	13.554	36.270	80.209
1280	N	GLU	164	14.206	40.675	78.643
1281	CA	GLU	164	13.574	41.302	77.477
1282	C	GLU	164	14.581	41.541	76.350
1283	O	GLU	164	14.200	41.505	75.174
1284	CB	GLU	164	12.941	42.623	77.900
1285	CG	GLU	164	12.141	43.250	76.761
1286	CD	GLU	164	11.519	44.562	77.226
1287	OE1	GLU	164	11.265	45.410	76.383
1288	OE2	GLU	164	11.350	44.704	78.430
1289	N	LEU	165	15.861	41.509	76.684
1290	CA	LEU	165	16.895	41.657	75.664
1291	C	LEU	165	17.027	40.353	74.890
1292	O	LEU	165	16.863	40.359	73.663
1293	CB	LEU	165	18.215	41.992	76.349
1294	CG	LEU	165	19.345	42.167	75.341
1295	CD1	LEU	165	19.026	43.276	74.344
1296	CD2	LEU	165	20.665	42.446	76.051
1297	N	ALA	166	16.953	39.246	75.610
1298	CA	ALA	166	17.028	37.932	74.974
1299	C	ALA	166	15.740	37.591	74.232
1300	O	ALA	166	15.827	37.059	73.117
1301	CB	ALA	166	17.297	36.885	76.047
1302	N	ALA	167	14.625	38.147	74.684
1303	CA	ALA	167	13.340	37.960	74.001

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1304	C	ALA	167	13.148	38.884	72.795
1305	O	ALA	167	12.249	38.657	71.979
1306	CB	ALA	167	12.220	38.184	75.009
1307	N	ALA	168	14.013	39.871	72.638
1308	CA	ALA	168	14.018	40.641	71.397
1309	C	ALA	168	15.080	40.104	70.441
1310	O	ALA	168	14.871	40.098	69.222
1311	CB	ALA	168	14.306	42.101	71.724
1312	N	ASP	169	16.103	39.480	71.001
1313	CA	ASP	169	17.201	38.928	70.197
1314	C	ASP	169	16.810	37.629	69.499
1315	O	ASP	169	16.684	37.606	68.268
1316	CB	ASP	169	18.397	38.644	71.106
1317	CG	ASP	169	18.972	39.917	71.726
1318	OD1	ASP	169	19.555	39.802	72.799
1319	OD2	ASP	169	18.909	40.956	71.083
1320	N	HIS	170	16.424	36.638	70.285
1321	CA	HIS	170	16.161	35.287	69.751
1322	C	HIS	170	14.990	35.251	68.759
1323	O	HIS	170	15.224	34.833	67.618
1324	CB	HIS	170	15.896	34.276	70.868
1325	CG	HIS	170	17.014	33.923	71.831
1326	ND1	HIS	170	17.675	34.744	72.670
1327	CD2	HIS	170	17.502	32.659	72.045
1328	CE1	HIS	170	18.581	34.034	73.368
1329	NE2	HIS	170	18.467	32.743	72.986
1330	N	PRO	171	13.777	35.670	69.119
1331	CA	PRO	171	12.717	35.738	68.113
1332	C	PRO	171	12.791	36.918	67.139
1333	O	PRO	171	11.867	37.031	66.325
1334	CB	PRO	171	11.442	35.830	68.891
1335	CG	PRO	171	11.758	36.089	70.348
1336	CD	PRO	171	13.272	36.104	70.435
1337	N	LEU	172	13.833	37.740	67.164
1338	CA	LEU	172	13.857	38.986	66.383
1339	C	LEU	172	12.535	39.720	66.592
1340	O	LEU	172	11.685	39.770	65.691
1341	CB	LEU	172	14.083	38.653	64.910
1342	CG	LEU	172	14.336	39.903	64.073
1343	CD1	LEU	172	15.554	40.665	64.586
1344	CD2	LEU	172	14.507	39.541	62.602
1345	N	SER	173	12.314	40.050	67.858
1346	CA	SER	173	11.094	40.669	68.423
1347	C	SER	173	9.763	39.882	68.367
1348	O	SER	173	8.976	40.007	69.316

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1349	CB	SER	173	10.902	42.013	67.731
1350	OG	SER	173	12.087	42.769	67.939
1351	N	ARG	174	9.507	39.106	67.319
1352	CA	ARG	174	8.189	38.477	67.131
1353	C	ARG	174	8.115	37.518	65.933
1354	O	ARG	174	7.017	37.068	65.584
1355	CB	ARG	174	7.215	39.625	66.873
1356	CG	ARG	174	7.671	40.436	65.661
1357	CD	ARG	174	7.136	41.862	65.675
1358	NE	ARG	174	5.668	41.905	65.629
1359	CZ	ARG	174	5.001	42.998	65.254
1360	NH1	ARG	174	3.667	43.001	65.252
1361	NH2	ARG	174	5.672	44.097	64.898
1362	N	ASP	175	9.242	37.200	65.318
1363	CA	ASP	175	9.216	36.473	64.038
1364	C	ASP	175	9.816	35.060	64.077
1365	O	ASP	175	9.442	34.201	63.270
1366	CB	ASP	175	10.006	37.334	63.055
1367	CG	ASP	175	10.065	36.692	61.674
1368	OD1	ASP	175	9.008	36.417	61.125
1369	OD2	ASP	175	11.170	36.521	61.177
1370	N	ASN	176	10.741	34.825	64.988
1371	CA	ASN	176	11.434	33.530	65.061
1372	C	ASN	176	11.035	32.742	66.312
1373	O	ASN	176	10.750	33.324	67.363
1374	CB	ASN	176	12.937	33.818	65.045
1375	CG	ASN	176	13.766	32.537	65.043
1376	OD1	ASN	176	13.465	31.587	64.310
1377	ND2	ASN	176	14.789	32.524	65.878
1378	N	LYS	177	10.959	31.430	66.172
1379	CA	LYS	177	10.591	30.563	67.295
1380	C	LYS	177	11.763	30.244	68.223
1381	O	LYS	177	12.868	29.872	67.799
1382	CB	LYS	177	10.030	29.261	66.741
1383	CG	LYS	177	8.715	29.497	66.013
1384	CD	LYS	177	8.152	28.205	65.438
1385	CE	LYS	177	6.805	28.445	64.766
1386	NZ	LYS	177	6.273	27.202	64.187
1387	N	TRP	178	11.507	30.415	69.506
1388	CA	TRP	178	12.464	29.969	70.519
1389	C	TRP	178	11.800	28.972	71.470
1390	O	TRP	178	10.571	28.838	71.478
1391	CB	TRP	178	13.051	31.177	71.252
1392	CG	TRP	178	12.247	31.835	72.361
1393	CD1	TRP	178	10.918	31.672	72.695

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1394	CD2	TRP	178	12.774	32.803	73.291
1395	NE1	TRP	178	10.647	32.436	73.781
1396	CE2	TRP	178	11.741	33.120	74.174
1397	CE3	TRP	178	14.026	33.381	73.447
1398	CZ2	TRP	178	11.977	33.980	75.238
1399	CZ3	TRP	178	14.246	34.268	74.491
1400	CH2	TRP	178	13.231	34.560	75.389
1401	N	VAL	179	12.619	28.226	72.189
1402	CA	VAL	179	12.135	27.251	73.172
1403	C	VAL	179	12.735	27.553	74.542
1404	O	VAL	179	13.920	27.305	74.791
1405	CB	VAL	179	12.547	25.842	72.745
1406	CG1	VAL	179	12.237	24.816	73.829
1407	CG2	VAL	179	11.886	25.429	71.439
1408	N	VAL	180	11.928	28.116	75.419
1409	CA	VAL	180	12.413	28.355	76.780
1410	C	VAL	180	11.946	27.269	77.751
1411	O	VAL	180	10.752	27.135	78.048
1412	CB	VAL	180	12.012	29.756	77.221
1413	CG1	VAL	180	13.004	30.787	76.709
1414	CG2	VAL	180	10.606	30.113	76.771
1415	N	GLN	181	12.906	26.470	78.191
1416	CA	GLN	181	12.617	25.338	79.083
1417	C	GLN	181	12.870	25.649	80.556
1418	O	GLN	181	13.510	26.644	80.918
1419	CB	GLN	181	13.419	24.098	78.687
1420	CG	GLN	181	12.956	23.514	77.354
1421	CD	GLN	181	13.411	22.060	77.205
1422	OE1	GLN	181	13.459	21.309	78.187
1423	NE2	GLN	181	13.688	21.668	75.971
1424	N	LYS	182	12.249	24.844	81.398
1425	CA	LYS	182	12.460	24.938	82.850
1426	C	LYS	182	13.832	24.403	83.252
1427	O	LYS	182	14.259	23.326	82.822
1428	CB	LYS	182	11.382	24.127	83.559
1429	CG	LYS	182	9.988	24.674	83.274
1430	CD	LYS	182	9.727	26.010	83.961
1431	CE	LYS	182	9.599	25.848	85.471
1432	NZ	LYS	182	8.465	24.976	85.815
1433	N	TYR	183	14.508	25.167	84.088
1434	CA	TYR	183	15.828	24.776	84.592
1435	C	TYR	183	15.746	24.009	85.910
1436	O	TYR	183	15.354	24.551	86.951
1437	CB	TYR	183	16.630	26.057	84.781
1438	CG	TYR	183	17.969	25.958	85.505

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1439	CD1	TYR	183	19.118	25.606	84.810
1440	CD2	TYR	183	18.039	26.233	86.865
1441	CE1	TYR	183	20.337	25.554	85.470
1442	CE2	TYR	183	19.257	26.184	87.526
1443	CZ	TYR	183	20.405	25.854	86.823
1444	OH	TYR	183	21.630	25.936	87.447
1445	N	ILE	184	16.114	22.742	85.849
1446	CA	ILE	184	16.281	21.949	87.071
1447	C	ILE	184	17.529	22.440	87.801
1448	O	ILE	184	18.629	22.458	87.240
1449	CB	ILE	184	16.400	20.474	86.690
1450	CG1	ILE	184	15.107	19.987	86.046
1451	CG2	ILE	184	16.747	19.591	87.886
1452	CD1	ILE	184	15.177	18.493	85.756
1453	N	GLU	185	17.364	22.738	89.080
1454	CA	GLU	185	18.420	23.390	89.870
1455	C	GLU	185	19.497	22.471	90.460
1456	O	GLU	185	20.215	22.890	91.375
1457	CB	GLU	185	17.761	24.213	90.967
1458	CG	GLU	185	16.949	25.344	90.347
1459	CD	GLU	185	16.406	26.270	91.428
1460	OE1	GLU	185	16.539	27.478	91.258
1461	OE2	GLU	185	15.920	25.762	92.428
1462	N	THR	186	19.631	21.255	89.953
1463	CA	THR	186	20.743	20.393	90.391
1464	C	THR	186	21.866	20.132	89.351
1465	O	THR	186	22.472	19.060	89.468
1466	CB	THR	186	20.152	19.042	90.777
1467	OG1	THR	186	19.602	18.445	89.609
1468	CG2	THR	186	19.051	19.171	91.822
1469	N	PRO	187	22.266	21.049	88.466
1470	CA	PRO	187	23.021	20.631	87.278
1471	C	PRO	187	24.545	20.716	87.427
1472	O	PRO	187	25.247	20.806	86.411
1473	CB	PRO	187	22.566	21.573	86.218
1474	CG	PRO	187	22.066	22.829	86.897
1475	CD	PRO	187	21.995	22.492	88.374
1476	N	LEU	188	25.046	20.613	88.649
1477	CA	LEU	188	26.482	20.774	88.913
1478	C	LEU	188	27.239	19.448	88.780
1479	O	LEU	188	28.469	19.417	88.649
1480	CB	LEU	188	26.629	21.307	90.335
1481	CG	LEU	188	28.018	21.878	90.587
1482	CD1	LEU	188	28.256	23.095	89.699
1483	CD2	LEU	188	28.192	22.251	92.055

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1484	N	LEU	189	26.490	18.363	88.674
1485	CA	LEU	189	27.101	17.033	88.529
1486	C	LEU	189	27.239	16.592	87.068
1487	O	LEU	189	27.461	15.406	86.795
1488	CB	LEU	189	26.319	15.997	89.334
1489	CG	LEU	189	26.951	15.724	90.703
1490	CD1	LEU	189	28.420	15.346	90.551
1491	CD2	LEU	189	26.819	16.893	91.677
1492	N	ILE	190	27.054	17.527	86.151
1493	CA	ILE	190	27.236	17.253	84.724
1494	C	ILE	190	28.206	18.242	84.092
1495	O	ILE	190	28.659	18.018	82.965
1496	CB	ILE	190	25.875	17.353	84.057
1497	CG1	ILE	190	24.976	18.280	84.855
1498	CG2	ILE	190	25.240	15.990	83.913
1499	CD1	ILE	190	23.514	18.142	84.467
1500	N	CYS	191	28.340	19.373	84.771
1501	CA	CYS	191	29.255	20.487	84.468
1502	C	CYS	191	28.779	21.644	85.337
1503	O	CYS	191	28.156	21.397	86.375
1504	CB	CYS	191	29.300	20.872	82.987
1505	SG	CYS	191	27.733	21.239	82.172
1506	N	ASP	192	28.994	22.878	84.913
1507	CA	ASP	192	28.500	24.007	85.710
1508	C	ASP	192	26.989	24.136	85.559
1509	O	ASP	192	26.262	24.276	86.552
1510	CB	ASP	192	29.168	25.298	85.245
1511	CG	ASP	192	28.651	26.480	86.063
1512	OD1	ASP	192	28.974	26.513	87.242
1513	OD2	ASP	192	27.783	27.180	85.560
1514	N	THR	193	26.520	24.013	84.328
1515	CA	THR	193	25.083	24.098	84.071
1516	C	THR	193	24.697	23.173	82.927
1517	O	THR	193	24.786	23.556	81.755
1518	CB	THR	193	24.714	25.536	83.730
1519	OG1	THR	193	25.101	26.369	84.814
1520	CG2	THR	193	23.219	25.718	83.504
1521	N	LYS	194	24.271	21.979	83.323
1522	CA	LYS	194	23.742	20.875	82.483
1523	C	LYS	194	24.463	20.569	81.166
1524	O	LYS	194	24.730	21.439	80.337
1525	CB	LYS	194	22.223	21.004	82.318
1526	CG	LYS	194	21.682	22.385	81.949
1527	CD	LYS	194	21.977	22.783	80.511
1528	CE	LYS	194	21.331	24.112	80.169

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1529	NZ	LYS	194	19.875	23.968	80.175
1530	N	PHE	195	24.811	19.304	81.002
1531	CA	PHE	195	25.551	18.901	79.804
1532	C	PHE	195	24.770	17.961	78.892
1533	O	PHE	195	24.930	18.055	77.669
1534	CB	PHE	195	26.814	18.173	80.267
1535	CG	PHE	195	27.777	17.742	79.158
1536	CD1	PHE	195	27.475	16.671	78.324
1537	CD2	PHE	195	28.980	18.406	78.998
1538	CE1	PHE	195	28.337	16.290	77.308
1539	CE2	PHE	195	29.847	18.019	77.989
1540	CZ	PHE	195	29.527	16.974	77.136
1541	N	ASP	196	23.881	17.162	79.475
1542	CA	ASP	196	23.356	15.944	78.818
1543	C	ASP	196	24.576	15.042	78.626
1544	O	ASP	196	25.444	15.111	79.500
1545	CB	ASP	196	22.684	16.309	77.495
1546	CG	ASP	196	21.517	15.411	77.099
1547	OD1	ASP	196	21.741	14.212	76.993
1548	OD2	ASP	196	20.585	15.969	76.543
1549	N	ILE	197	24.488	14.022	77.784
1550	CA	ILE	197	25.659	13.271	77.267
1551	C	ILE	197	25.279	12.821	75.865
1552	O	ILE	197	24.134	12.398	75.694
1553	CB	ILE	197	25.998	12.024	78.095
1554	CG1	ILE	197	26.420	12.354	79.510
1555	CG2	ILE	197	27.144	11.240	77.458
1556	CD1	ILE	197	27.749	13.096	79.506
1557	N	ARG	198	26.183	12.923	74.899
1558	CA	ARG	198	25.862	12.535	73.523
1559	C	ARG	198	25.479	11.064	73.441
1560	O	ARG	198	26.242	10.161	73.808
1561	CB	ARG	198	27.056	12.831	72.622
1562	CG	ARG	198	26.689	12.644	71.155
1563	CD	ARG	198	25.500	13.514	70.751
1564	NE	ARG	198	25.764	14.948	70.943
1565	CZ	ARG	198	25.356	15.898	70.100
1566	NH1	ARG	198	24.809	15.563	68.930
1567	NH2	ARG	198	25.581	17.182	70.387
1568	N	GLN	199	24.242	10.872	73.017
1569	CA	GLN	199	23.637	9.547	72.950
1570	C	GLN	199	23.805	8.938	71.566
1571	O	GLN	199	24.034	9.659	70.585
1572	CB	GLN	199	22.151	9.707	73.261
1573	CG	GLN	199	21.936	10.696	74.405



Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1574	CD	GLN	199	21.540	10.019	75.712
1575	OE1	GLN	199	20.423	9.500	75.818
1576	NE2	GLN	199	22.339	10.239	76.739
1577	N	TRP	200	23.797	7.617	71.518
1578	CA	TRP	200	23.790	6.907	70.229
1579	C	TRP	200	23.221	5.491	70.356
1580	O	TRP	200	23.968	4.530	70.591
1581	CB	TRP	200	25.218	6.841	69.701
1582	CG	TRP	200	25.359	6.237	68.316
1583	CD1	TRP	200	26.122	5.144	67.970
1584	CD2	TRP	200	24.731	6.702	67.103
1585	NE1	TRP	200	25.990	4.934	66.638
1586	CE2	TRP	200	25.171	5.841	66.077
1587	CE3	TRP	200	23.867	7.746	66.814
1588	CZ2	TRP	200	24.735	6.044	64.777
1589	CZ3	TRP	200	23.434	7.940	65.508
1590	CH2	TRP	200	23.867	7.092	64.493
1591	N	PHE	201	21.913	5.359	70.182
1592	CA	PHE	201	21.301	4.024	70.300
1593	C	PHE	201	21.163	3.327	68.960
1594	O	PHE	201	20.784	3.920	67.943
1595	CB	PHE	201	19.970	4.036	71.054
1596	CG	PHE	201	18.855	4.956	70.570
1597	CD1	PHE	201	18.049	4.583	69.502
1598	CD2	PHE	201	18.623	6.156	71.230
1599	CE1	PHE	201	17.020	5.418	69.088
1600	CE2	PHE	201	17.592	6.987	70.819
1601	CZ	PHE	201	16.792	6.617	69.748
1602	N	LEU	202	21.338	2.022	69.018
1603	CA	LEU	202	21.421	1.218	67.799
1604	C	LEU	202	20.087	0.538	67.506
1605	O	LEU	202	19.979	-0.692	67.610
1606	CB	LEU	202	22.529	0.162	67.932
1607	CG	LEU	202	23.967	0.705	67.955
1608	CD1	LEU	202	24.162	1.819	66.940
1609	CD2	LEU	202	24.449	1.163	69.331
1610	N	VAL	203	19.101	1.349	67.140
1611	CA	VAL	203	17.736	0.888	66.815
1612	C	VAL	203	17.208	-0.058	67.892
1613	O	VAL	203	17.353	-1.284	67.784
1614	CB	VAL	203	17.785	0.204	65.450
1615	CG1	VAL	203	16.421	-0.319	65.014
1616	CG2	VAL	203	18.335	1.159	64.398
1617	N	THR	204	16.773	0.540	68.995
1618	CA	THR	204	16.378	-0.163	70.241

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1619	C	THR	204	17.535	-0.822	71.031
1620	O	THR	204	17.454	-0.883	72.263
1621	CB	THR	204	15.271	-1.174	69.919
1622	OG1	THR	204	14.078	-0.433	69.704
1623	CG2	THR	204	14.989	-2.150	71.058
1624	N	ASP	205	18.637	-1.175	70.382
1625	CA	ASP	205	19.786	-1.756	71.086
1626	C	ASP	205	20.497	-0.727	71.960
1627	O	ASP	205	20.904	0.354	71.506
1628	CB	ASP	205	20.781	-2.317	70.074
1629	CG	ASP	205	20.187	-3.460	69.254
1630	OD1	ASP	205	20.841	-3.845	68.294
1631	OD2	ASP	205	19.303	-4.117	69.788
1632	N	TRP	206	20.602	-1.080	73.229
1633	CA	TRP	206	21.315	-0.260	74.210
1634	C	TRP	206	22.788	-0.650	74.303
1635	O	TRP	206	23.140	-1.737	74.772
1636	CB	TRP	206	20.625	-0.443	75.562
1637	CG	TRP	206	21.440	-0.056	76.784
1638	CD1	TRP	206	21.855	1.206	77.159
1639	CD2	TRP	206	21.933	-0.965	77.791
1640	NE1	TRP	206	22.566	1.098	78.314
1641	CE2	TRP	206	22.633	-0.182	78.725
1642	CE3	TRP	206	21.839	-2.337	77.957
1643	CZ2	TRP	206	23.232	-0.789	79.820
1644	CZ3	TRP	206	22.446	-2.940	79.053
1645	CH2	TRP	206	23.138	-2.169	79.981
1646	N	ASN	207	23.632	0.223	73.779
1647	CA	ASN	207	25.082	0.083	73.944
1648	C	ASN	207	25.456	0.542	75.351
1649	O	ASN	207	25.306	1.722	75.693
1650	CB	ASN	207	25.773	0.940	72.886
1651	CG	ASN	207	27.284	0.711	72.885
1652	OD1	ASN	207	27.785	-0.259	72.307
1653	ND2	ASN	207	28.000	1.658	73.461
1654	N	PRO	208	26.058	-0.369	76.102
1655	CA	PRO	208	25.981	-0.344	77.575
1656	C	PRO	208	26.917	0.620	78.320
1657	O	PRO	208	26.930	0.587	79.555
1658	CB	PRO	208	26.307	-1.744	78.000
1659	CG	PRO	208	26.735	-2.563	76.797
1660	CD	PRO	208	26.542	-1.657	75.595
1661	N	LEU	209	27.643	1.486	77.634
1662	CA	LEU	209	28.696	2.230	78.338
1663	C	LEU	209	28.286	3.636	78.799

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1664	O	LEU	209	27.103	3.934	79.005
1665	CB	LEU	209	29.950	2.278	77.483
1666	CG	LEU	209	31.177	1.775	78.241
1667	CD1	LEU	209	31.024	0.315	78.647
1668	CD2	LEU	209	32.441	1.972	77.414
1669	N	THR	210	29.300	4.471	78.970
1670	CA	THR	210	29.176	5.698	79.751
1671	C	THR	210	30.256	6.760	79.455
1672	O	THR	210	31.331	6.450	78.935
1673	CB	THR	210	29.317	5.195	81.174
1674	OG1	THR	210	29.616	6.282	82.030
1675	CG2	THR	210	30.445	4.182	81.264
1676	N	ILE	211	29.937	8.008	79.779
1677	CA	ILE	211	30.884	9.134	79.730
1678	C	ILE	211	30.384	10.318	80.572
1679	O	ILE	211	29.178	10.593	80.582
1680	CB	ILE	211	31.084	9.508	78.263
1681	CG1	ILE	211	32.522	9.249	77.852
1682	CG2	ILE	211	30.704	10.953	77.970
1683	CD1	ILE	211	33.485	10.145	78.617
1684	N	TRP	212	31.265	10.865	81.402
1685	CA	TRP	212	30.932	12.008	82.271
1686	C	TRP	212	31.738	13.277	82.063
1687	O	TRP	212	32.701	13.288	81.296
1688	CB	TRP	212	30.968	11.549	83.722
1689	CG	TRP	212	29.622	10.906	83.891
1690	CD1	TRP	212	29.285	9.615	84.207
1691	CD2	TRP	212	28.400	11.628	83.730
1692	NE1	TRP	212	27.948	9.493	84.035
1693	CE2	TRP	212	27.400	10.662	83.650
1694	CE3	TRP	212	28.123	12.964	83.424
1695	CZ2	TRP	212	26.169	11.000	83.100
1696	CZ3	TRP	212	26.867	13.303	82.961
1697	CH2	TRP	212	25.910	12.330	82.777
1698	N	PHE	213	31.185	14.371	82.576
1699	CA	PHE	213	31.766	15.722	82.432
1700	C	PHE	213	31.444	16.584	83.655
1701	O	PHE	213	31.410	17.816	83.560
1702	CB	PHE	213	31.151	16.387	81.199
1703	CG	PHE	213	31.538	15.794	79.850
1704	CD1	PHE	213	30.801	14.751	79.301
1705	CD2	PHE	213	32.606	16.326	79.147
1706	CE1	PHE	213	31.166	14.217	78.070
1707	CE2	PHE	213	32.969	15.795	77.923
1708	CZ	PHE	213	32.254	14.739	77.386

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1709	N	TYR	214	31.342	15.925	84.799
1710	CA	TYR	214	30.822	16.510	86.052
1711	C	TYR	214	31.707	17.598	86.670
1712	O	TYR	214	32.231	18.443	85.941
1713	CB	TYR	214	30.541	15.373	87.046
1714	CG	TYR	214	31.679	14.448	87.518
1715	CD1	TYR	214	32.041	14.475	88.859
1716	CD2	TYR	214	32.293	13.549	86.652
1717	CE1	TYR	214	33.049	13.642	89.325
1718	CE2	TYR	214	33.303	12.716	87.114
1719	CZ	TYR	214	33.681	12.767	88.450
1720	OH	TYR	214	34.685	11.950	88.909
1721	N	LYS	215	31.645	17.756	87.978
1722	CA	LYS	215	32.567	18.693	88.630
1723	C	LYS	215	33.276	18.063	89.825
1724	O	LYS	215	32.659	17.606	90.795
1725	CB	LYS	215	31.845	19.980	88.997
1726	CG	LYS	215	32.275	21.074	88.028
1727	CD	LYS	215	31.487	22.359	88.204
1728	CE	LYS	215	32.103	23.477	87.379
1729	NZ	LYS	215	32.300	23.069	85.982
1730	N	THR	263	36.916	26.099	84.721
1731	CA	THR	263	36.173	26.956	83.787
1732	C	THR	263	34.709	27.074	84.203
1733	O	THR	263	33.842	27.322	83.353
1734	CB	THR	263	36.230	26.367	82.387
1735	OG1	THR	263	35.590	25.100	82.431
1736	CG2	THR	263	37.661	26.185	81.893
1737	N	SER	264	34.488	27.138	85.508
1738	CA	SER	264	33.128	27.122	86.088
1739	C	SER	264	32.314	28.415	85.933
1740	O	SER	264	31.167	28.460	86.386
1741	CB	SER	264	33.245	26.816	87.577
1742	OG	SER	264	33.957	27.880	88.191
1743	N	THR	265	32.840	29.403	85.225
1744	CA	THR	265	32.115	30.660	85.014
1745	C	THR	265	31.146	30.573	83.834
1746	O	THR	265	30.357	31.499	83.619
1747	CB	THR	265	33.122	31.766	84.727
1748	OG1	THR	265	33.709	31.516	83.457
1749	CG2	THR	265	34.223	31.814	85.780
1750	N	ARG	266	31.222	29.501	83.059
1751	CA	ARG	266	30.264	29.332	81.962
1752	C	ARG	266	29.095	28.425	82.334
1753	O	ARG	266	29.258	27.237	82.643

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1754	CB	ARG	266	30.982	28.818	80.725
1755	CG	ARG	266	31.777	29.945	80.081
1756	CD	ARG	266	32.570	29.457	78.880
1757	NE	ARG	266	33.633	28.544	79.315
1758	CZ	ARG	266	34.507	27.983	78.478
1759	NH1	ARG	266	34.399	28.187	77.163
1760	NH2	ARG	266	35.463	27.186	78.956
1761	N	PHE	267	27.913	28.969	82.100
1762	CA	PHE	267	26.638	28.301	82.399
1763	C	PHE	267	26.120	27.416	81.259
1764	O	PHE	267	24.926	27.457	80.942
1765	CB	PHE	267	25.600	29.381	82.724
1766	CG	PHE	267	25.509	30.561	81.745
1767	CD1	PHE	267	24.863	30.422	80.522
1768	CD2	PHE	267	26.058	31.790	82.096
1769	CE1	PHE	267	24.785	31.496	79.645
1770	CE2	PHE	267	25.980	32.865	81.220
1771	CZ	PHE	267	25.346	32.718	79.993
1772	N	GLN	268	26.982	26.594	80.688
1773	CA	GLN	268	26.590	25.847	79.494
1774	C	GLN	268	27.075	24.393	79.455
1775	O	GLN	268	27.784	23.909	80.347
1776	CB	GLN	268	27.077	26.629	78.286
1777	CG	GLN	268	25.937	27.324	77.550
1778	CD	GLN	268	25.853	26.690	76.169
1779	OE1	GLN	268	25.869	25.453	76.058
1780	NE2	GLN	268	25.892	27.533	75.152
1781	N	GLU	269	26.843	23.808	78.288
1782	CA	GLU	269	26.862	22.355	78.078
1783	C	GLU	269	28.123	21.792	77.397
1784	O	GLU	269	29.249	22.191	77.713
1785	CB	GLU	269	25.615	22.140	77.227
1786	CG	GLU	269	24.486	22.975	77.843
1787	CD	GLU	269	23.397	23.320	76.845
1788	OE1	GLU	269	23.710	23.280	75.666
1789	OE2	GLU	269	22.244	23.400	77.243
1790	N	TYR	270	27.884	20.878	76.460
1791	CA	TYR	270	28.895	20.054	75.737
1792	C	TYR	270	30.297	20.611	75.493
1793	O	TYR	270	30.462	21.794	75.181
1794	CB	TYR	270	28.360	19.602	74.388
1795	CG	TYR	270	27.349	18.465	74.383
1796	CD1	TYR	270	26.027	18.716	74.714
1797	CD2	TYR	270	27.741	17.183	74.018
1798	CE1	TYR	270	25.100	17.688	74.697

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1799	CE2	TYR	270	26.810	16.152	74.007
1800	CZ	TYR	270	25.490	16.411	74.349
1801	OH	TYR	270	24.545	15.410	74.343
1802	N	LEU	271	31.140	19.633	75.190
1803	CA	LEU	271	32.612	19.726	75.218
1804	C	LEU	271	33.330	20.217	73.966
1805	O	LEU	271	34.568	20.213	73.948
1806	CB	LEU	271	33.179	18.337	75.540
1807	CG	LEU	271	33.191	17.350	74.363
1808	CD1	LEU	271	34.374	16.395	74.483
1809	CD2	LEU	271	31.882	16.579	74.182
1810	N	GLN	272	32.619	20.693	72.961
1811	CA	GLN	272	33.300	20.916	71.681
1812	C	GLN	272	34.090	22.217	71.637
1813	O	GLN	272	35.085	22.296	70.905
1814	CB	GLN	272	32.254	20.909	70.574
1815	CG	GLN	272	32.874	21.007	69.188
1816	CD	GLN	272	31.769	21.016	68.146
1817	OE1	GLN	272	30.621	21.359	68.448
1818	NE2	GLN	272	32.132	20.658	66.928
1819	N	ARG	273	33.742	23.184	72.469
1820	CA	ARG	273	34.474	24.443	72.419
1821	C	ARG	273	35.527	24.495	73.523
1822	O	ARG	273	36.609	25.056	73.295
1823	CB	ARG	273	33.444	25.570	72.482
1824	CG	ARG	273	32.416	25.302	71.375
1825	CD	ARG	273	31.165	26.163	71.446
1826	NE	ARG	273	31.428	27.556	71.060
1827	CZ	ARG	273	30.458	28.470	70.983
1828	NH1	ARG	273	30.753	29.730	70.657
1829	NH2	ARG	273	29.197	28.129	71.260
1830	N	GLN	274	35.281	23.771	74.609
1831	CA	GLN	274	36.265	23.595	75.702
1832	C	GLN	274	35.825	22.531	76.693
1833	O	GLN	274	35.372	22.928	77.772
1834	CB	GLN	274	36.373	24.881	76.529
1835	CG	GLN	274	37.398	25.889	76.030
1836	CD	GLN	274	38.778	25.265	76.001
1837	OE1	GLN	274	39.326	24.872	77.039
1838	NE2	GLN	274	39.279	25.113	74.790
1839	N	GLY	275	36.071	21.254	76.436
1840	CA	GLY	275	35.565	20.212	77.349
1841	C	GLY	275	36.568	19.176	77.871
1842	O	GLY	275	37.733	19.130	77.464
1843	N	ARG	276	36.090	18.407	78.841

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1844	CA	ARG	276	36.853	17.313	79.479
1845	C	ARG	276	35.913	16.208	80.000
1846	O	ARG	276	34.974	16.515	80.741
1847	CB	ARG	276	37.674	17.950	80.613
1848	CG	ARG	276	38.069	16.973	81.720
1849	CD	ARG	276	37.046	16.965	82.851
1850	NE	ARG	276	37.202	15.762	83.664
1851	CZ	ARG	276	36.214	15.000	84.130
1852	NH1	ARG	276	34.945	15.257	83.809
1853	NH2	ARG	276	36.509	13.966	84.917
1854	N	GLY	277	36.227	14.946	79.736
1855	CA	GLY	277	35.329	13.852	80.149
1856	C	GLY	277	35.904	12.839	81.142
1857	O	GLY	277	36.959	13.051	81.749
1858	N	ALA	278	35.128	11.787	81.370
1859	CA	ALA	278	35.488	10.667	82.266
1860	C	ALA	278	34.484	9.517	82.209
1861	O	ALA	278	33.380	9.635	82.746
1862	CB	ALA	278	35.460	11.135	83.711
1863	N	VAL	279	34.884	8.390	81.652
1864	CA	VAL	279	33.983	7.230	81.579
1865	C	VAL	279	33.850	6.557	82.950
1866	O	VAL	279	34.852	6.317	83.635
1867	CB	VAL	279	34.567	6.241	80.577
1868	CG1	VAL	279	33.615	5.096	80.286
1869	CG2	VAL	279	34.918	6.945	79.284
1870	N	TRP	280	32.615	6.329	83.366
1871	CA	TRP	280	32.362	5.680	84.654
1872	C	TRP	280	31.191	4.700	84.537
1873	O	TRP	280	31.386	3.521	84.208
1874	CB	TRP	280	32.064	6.747	85.703
1875	CG	TRP	280	32.847	6.545	86.981
1876	CD1	TRP	280	33.985	7.224	87.357
1877	CD2	TRP	280	32.557	5.603	88.037
1878	NE1	TRP	280	34.392	6.742	88.558
1879	CE2	TRP	280	33.573	5.768	88.996
1880	CE3	TRP	280	31.559	4.661	88.227
1881	CZ2	TRP	280	33.585	4.967	90.126
1882	CZ3	TRP	280	31.571	3.871	89.372
1883	CH2	TRP	280	32.582	4.020	90.314
1884	N	GLY	281	29.993	5.179	84.844
1885	CA	GLY	281	28.784	4.362	84.666
1886	C	GLY	281	27.594	5.213	84.226
1887	O	GLY	281	27.708	6.436	84.075
1888	N	SER	282	26.440	4.567	84.130

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1889	CA	SER	282	25.162	5.232	83.779
1890	C	SER	282	24.367	5.610	85.025
1891	O	SER	282	23.137	5.603	85.049
1892	CB	SER	282	24.373	4.269	82.904
1893	OG	SER	282	25.149	3.995	81.748
1894	N	VAL	283	25.108	6.198	85.936
1895	CA	VAL	283	24.798	6.259	87.359
1896	C	VAL	283	23.789	7.309	87.865
1897	O	VAL	283	22.854	7.680	87.146
1898	CB	VAL	283	26.180	6.391	87.944
1899	CG1	VAL	283	26.851	5.026	87.945
1900	CG2	VAL	283	26.987	7.330	87.056
1901	N	ILE	284	23.872	7.601	89.158
1902	CA	ILE	284	22.805	8.318	89.882
1903	C	ILE	284	23.179	9.781	90.210
1904	O	ILE	284	23.313	10.582	89.276
1905	CB	ILE	284	22.478	7.503	91.137
1906	CG1	ILE	284	22.653	6.016	90.847
1907	CG2	ILE	284	21.035	7.721	91.606
1908	CD1	ILE	284	22.223	5.150	92.023
1909	N	TYR	285	23.248	10.135	91.494
1910	CA	TYR	285	23.580	11.504	91.980
1911	C	TYR	285	22.560	12.583	91.540
1912	O	TYR	285	21.772	12.317	90.628
1913	CB	TYR	285	25.020	11.816	91.582
1914	CG	TYR	285	26.090	11.349	92.573
1915	CD1	TYR	285	25.762	10.515	93.636
1916	CD2	TYR	285	27.400	11.791	92.422
1917	CE1	TYR	285	26.745	10.098	94.522
1918	CE2	TYR	285	28.386	11.372	93.305
1919	CZ	TYR	285	28.055	10.520	94.349
1920	OH	TYR	285	29.044	10.043	95.183
1921	N	PRO	286	22.557	13.793	92.106
1922	CA	PRO	286	23.764	14.565	92.505
1923	C	PRO	286	24.390	14.143	93.835
1924	O	PRO	286	25.616	14.182	93.989
1925	CB	PRO	286	23.328	15.995	92.590
1926	CG	PRO	286	21.841	16.088	92.332
1927	CD	PRO	286	21.400	14.687	91.951
1928	N	SER	287	23.552	13.778	94.786
1929	CA	SER	287	24.020	13.201	96.048
1930	C	SER	287	22.937	12.269	96.558
1931	O	SER	287	23.183	11.300	97.285
1932	CB	SER	287	24.258	14.300	97.081
1933	OG	SER	287	25.335	15.121	96.661



Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1934	N	MET	288	21.727	12.602	96.148
1935	CA	MET	288	20.536	11.884	96.590
1936	C	MET	288	20.171	10.737	95.661
1937	O	MET	288	20.659	10.632	94.529
1938	CB	MET	288	19.376	12.866	96.699
1939	CG	MET	288	19.177	13.429	98.111
1940	SD	MET	288	20.515	14.389	98.870
1941	CE	MET	288	21.344	13.099	99.832
1942	N	LYS	289	19.346	9.860	96.204
1943	CA	LYS	289	18.858	8.682	95.484
1944	C	LYS	289	17.475	8.943	94.894
1945	O	LYS	289	17.365	9.159	93.681
1946	CB	LYS	289	18.783	7.551	96.508
1947	CG	LYS	289	18.269	6.235	95.932
1948	CD	LYS	289	18.028	5.241	97.063
1949	CE	LYS	289	17.433	3.931	96.562
1950	NZ	LYS	289	17.166	3.020	97.687
1951	N	LYS	290	16.542	9.257	95.788
1952	CA	LYS	290	15.095	9.383	95.497
1953	C	LYS	290	14.651	8.816	94.140
1954	O	LYS	290	14.882	7.638	93.851
1955	CB	LYS	290	14.673	10.844	95.684
1956	CG	LYS	290	15.700	11.857	95.167
1957	CD	LYS	290	15.222	13.289	95.384
1958	CE	LYS	290	13.900	13.560	94.670
1959	NZ	LYS	290	14.031	13.416	93.212
1960	N	ALA	291	13.998	9.636	93.333
1961	CA	ALA	291	13.525	9.202	92.012
1962	C	ALA	291	14.541	9.450	90.893
1963	O	ALA	291	14.182	9.403	89.710
1964	CB	ALA	291	12.234	9.947	91.693
1965	N	ILE	292	15.779	9.741	91.254
1966	CA	ILE	292	16.765	10.170	90.263
1967	C	ILE	292	17.585	9.001	89.722
1968	O	ILE	292	18.076	8.158	90.482
1969	CB	ILE	292	17.696	11.182	90.939
1970	CG1	ILE	292	16.913	12.361	91.497
1971	CG2	ILE	292	18.748	11.705	89.969
1972	CD1	ILE	292	16.287	13.188	90.377
1973	N	ALA	293	17.598	8.894	88.404
1974	CA	ALA	293	18.621	8.110	87.709
1975	C	ALA	293	18.570	6.582	87.793
1976	O	ALA	293	17.622	5.994	88.328
1977	CB	ALA	293	19.983	8.631	88.135
1978	N	HIS	294	19.680	5.998	87.353

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
1979	CA	HIS	294	19.873	4.576	86.992
1980	C	HIS	294	18.626	3.708	86.806
1981	O	HIS	294	18.082	3.653	85.695
1982	CB	HIS	294	20.812	3.953	88.003
1983	CG	HIS	294	22.051	3.422	87.323
1984	ND1	HIS	294	22.110	2.877	86.095
1985	CD2	HIS	294	23.329	3.423	87.818
1986	CE1	HIS	294	23.379	2.507	85.831
1987	NE2	HIS	294	24.133	2.844	86.898
1988	N	ALA	295	18.090	3.151	87.883
1989	CA	ALA	295	16.939	2.233	87.779
1990	C	ALA	295	15.670	2.857	87.185
1991	O	ALA	295	14.946	2.176	86.447
1992	CB	ALA	295	16.606	1.736	89.180
1993	N	MET	296	15.542	4.171	87.284
1994	CA	MET	296	14.376	4.866	86.734
1995	C	MET	296	14.478	5.083	85.218
1996	O	MET	296	13.447	5.121	84.535
1997	CB	MET	296	14.299	6.222	87.431
1998	CG	MET	296	13.028	6.981	87.072
1999	SD	MET	296	11.494	6.205	87.625
2000	CE	MET	296	11.815	6.216	89.405
2001	N	LYS	297	15.678	4.948	84.675
2002	CA	LYS	297	15.918	5.255	83.262
2003	C	LYS	297	15.701	4.080	82.340
2004	O	LYS	297	15.696	4.234	81.109
2005	CB	LYS	297	17.356	5.645	83.125
2006	CG	LYS	297	17.700	6.765	84.068
2007	CD	LYS	297	19.200	6.840	84.062
2008	CE	LYS	297	19.733	8.109	84.661
2009	NZ	LYS	297	21.174	7.974	84.702
2010	N	VAL	298	15.372	2.947	82.931
2011	CA	VAL	298	15.039	1.764	82.151
2012	C	VAL	298	13.695	1.957	81.436
2013	O	VAL	298	13.554	1.511	80.292
2014	CB	VAL	298	15.008	0.602	83.139
2015	CG1	VAL	298	14.678	-0.715	82.462
2016	CG2	VAL	298	16.341	0.495	83.873
2017	N	ALA	299	12.893	2.890	81.933
2018	CA	ALA	299	11.633	3.240	81.277
2019	C	ALA	299	11.813	4.184	80.081
2020	O	ALA	299	11.076	4.059	79.093
2021	CB	ALA	299	10.760	3.905	82.329
2022	N	GLN	300	12.885	4.965	80.075
2023	CA	GLN	300	13.200	5.793	78.903

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2024	C	GLN	300	13.700	4.903	77.781
2025	O	GLN	300	13.013	4.752	76.760
2026	CB	GLN	300	14.328	6.770	79.238
2027	CG	GLN	300	13.901	7.960	80.088
2028	CD	GLN	300	13.602	9.187	79.225
2029	OE1	GLN	300	13.397	9.072	78.008
2030	NE2	GLN	300	13.762	10.354	79.822
2031	N	ASP	301	14.644	4.055	78.164
2032	CA	ASP	301	15.382	3.209	77.222
2033	C	ASP	301	14.614	1.983	76.719
2034	O	ASP	301	15.062	1.343	75.762
2035	CB	ASP	301	16.649	2.767	77.949
2036	CG	ASP	301	17.689	2.175	77.001
2037	OD1	ASP	301	18.350	1.230	77.409
2038	OD2	ASP	301	17.839	2.702	75.907
2039	N	HIS	302	13.470	1.668	77.301
2040	CA	HIS	302	12.664	0.593	76.721
2041	C	HIS	302	11.460	1.077	75.922
2042	O	HIS	302	10.935	0.299	75.122
2043	CB	HIS	302	12.193	-0.352	77.816
2044	CG	HIS	302	13.272	-1.295	78.302
2045	ND1	HIS	302	13.304	-1.926	79.488
2046	CD2	HIS	302	14.395	-1.686	77.610
2047	CE1	HIS	302	14.415	-2.687	79.560
2048	NE2	HIS	302	15.090	-2.536	78.398
2049	N	VAL	303	11.052	2.327	76.068
2050	CA	VAL	303	9.872	2.760	75.313
2051	C	VAL	303	10.228	3.737	74.194
2052	O	VAL	303	9.809	3.556	73.040
2053	CB	VAL	303	8.876	3.391	76.280
2054	CG1	VAL	303	7.617	3.852	75.553
2055	CG2	VAL	303	8.513	2.431	77.406
2056	N	GLU	304	11.145	4.642	74.485
2057	CA	GLU	304	11.525	5.670	73.503
2058	C	GLU	304	12.277	5.126	72.264
2059	O	GLU	304	11.820	5.466	71.163
2060	CB	GLU	304	12.332	6.755	74.218
2061	CG	GLU	304	12.033	8.167	73.703
2062	CD	GLU	304	12.718	8.450	72.375
2063	OE1	GLU	304	13.748	7.828	72.133
2064	OE2	GLU	304	12.292	9.370	71.684
2065	N	PRO	305	13.301	4.272	72.362
2066	CA	PRO	305	13.986	3.827	71.138
2067	C	PRO	305	13.201	2.863	70.238
2068	O	PRO	305	13.701	2.557	69.153

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2069	CB	PRO	305	15.249	3.164	71.589
2070	CG	PRO	305	15.228	3.019	73.095
2071	CD	PRO	305	13.966	3.720	73.557
2072	N	ARG	306	11.990	2.461	70.603
2073	CA	ARG	306	11.194	1.629	69.702
2074	C	ARG	306	10.495	2.492	68.656
2075	O	ARG	306	10.490	2.124	67.474
2076	CB	ARG	306	10.148	0.874	70.508
2077	CG	ARG	306	10.782	0.029	71.603
2078	CD	ARG	306	9.725	-0.825	72.289
2079	NE	ARG	306	8.612	0.012	72.766
2080	CZ	ARG	306	7.880	-0.289	73.840
2081	NH1	ARG	306	6.855	0.491	74.188
2082	NH2	ARG	306	8.150	-1.390	74.545
2083	N	LYS	307	10.211	3.737	69.019
2084	CA	LYS	307	9.608	4.672	68.061
2085	C	LYS	307	10.672	5.130	67.075
2086	O	LYS	307	10.476	5.071	65.853
2087	CB	LYS	307	9.092	5.893	68.813
2088	CG	LYS	307	8.117	5.517	69.921
2089	CD	LYS	307	7.575	6.766	70.609
2090	CE	LYS	307	8.699	7.614	71.196
2091	NZ	LYS	307	8.166	8.840	71.812
2092	N	ASN	308	11.880	5.223	67.603
2093	CA	ASN	308	13.041	5.580	66.796
2094	C	ASN	308	13.749	4.373	66.178
2095	O	ASN	308	14.868	4.506	65.671
2096	CB	ASN	308	13.979	6.421	67.633
2097	CG	ASN	308	13.300	7.745	67.970
2098	OD1	ASN	308	12.343	8.167	67.307
2099	ND2	ASN	308	13.811	8.388	69.001
2100	N	SER	309	13.092	3.223	66.187
2101	CA	SER	309	13.511	2.113	65.336
2102	C	SER	309	12.789	2.221	64.001
2103	O	SER	309	13.301	1.767	62.972
2104	CB	SER	309	13.152	0.790	65.997
2105	OG	SER	309	13.971	0.656	67.146
2106	N	PHE	310	11.686	2.953	64.002
2107	CA	PHE	310	10.999	3.266	62.747
2108	C	PHE	310	11.547	4.587	62.219
2109	O	PHE	310	11.749	4.769	61.014
2110	CB	PHE	310	9.507	3.433	63.024
2111	CG	PHE	310	8.858	2.302	63.818
2112	CD1	PHE	310	8.222	2.586	65.020
2113	CD2	PHE	310	8.891	0.996	63.343

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2114	CE1	PHE	310	7.630	1.566	65.753
2115	CE2	PHE	310	8.300	-0.024	64.076
2116	CZ	PHE	310	7.671	0.260	65.282
2117	N	GLU	311	11.925	5.433	63.165
2118	CA	GLU	311	12.505	6.744	62.849
2119	C	GLU	311	14.033	6.753	62.710
2120	O	GLU	311	14.591	7.803	62.383
2121	CB	GLU	311	12.096	7.722	63.943
2122	CG	GLU	311	10.585	7.914	64.021
2123	CD	GLU	311	10.045	8.463	62.703
2124	OE1	GLU	311	9.522	7.669	61.933
2125	OE2	GLU	311	10.125	9.666	62.509
2126	N	LEU	312	14.673	5.609	62.913
2127	CA	LEU	312	16.139	5.410	62.804
2128	C	LEU	312	17.033	6.607	63.165
2129	O	LEU	312	17.841	7.028	62.326
2130	CB	LEU	312	16.433	5.004	61.365
2131	CG	LEU	312	15.697	3.727	60.972
2132	CD1	LEU	312	15.764	3.496	59.467
2133	CD2	LEU	312	16.237	2.520	61.730
2134	N	TYR	313	16.952	7.067	64.410
2135	CA	TYR	313	17.764	8.212	64.895
2136	C	TYR	313	17.453	8.514	66.367
2137	O	TYR	313	16.573	7.872	66.949
2138	CB	TYR	313	17.521	9.465	64.036
2139	CG	TYR	313	16.247	10.300	64.246
2140	CD1	TYR	313	15.074	9.748	64.747
2141	CD2	TYR	313	16.290	11.650	63.922
2142	CE1	TYR	313	13.950	10.542	64.928
2143	CE2	TYR	313	15.166	12.446	64.097
2144	CZ	TYR	313	13.997	11.889	64.600
2145	OH	TYR	313	12.862	12.655	64.716
2146	N	GLY	314	18.149	9.468	66.966
2147	CA	GLY	314	17.768	9.882	68.324
2148	C	GLY	314	18.922	10.054	69.306
2149	O	GLY	314	19.587	9.076	69.678
2150	N	ALA	315	19.063	11.273	69.808
2151	CA	ALA	315	20.085	11.542	70.826
2152	C	ALA	315	19.905	12.831	71.636
2153	O	ALA	315	20.075	13.922	71.073
2154	CB	ALA	315	21.434	11.607	70.123
2155	N	ASP	316	19.530	12.674	72.907
2156	CA	ASP	316	19.681	13.679	74.014
2157	C	ASP	316	18.537	13.661	75.037
2158	O	ASP	316	17.411	14.138	74.804

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2159	CB	ASP	316	19.997	15.109	73.586
2160	CG	ASP	316	21.507	15.237	73.351
2161	OD1	ASP	316	22.163	14.220	73.214
2162	OD2	ASP	316	22.002	16.356	73.434
2163	N	PHE	317	18.888	13.152	76.210
2164	CA	PHE	317	17.938	12.996	77.318
2165	C	PHE	317	18.437	13.647	78.623
2166	O	PHE	317	19.633	13.662	78.942
2167	CB	PHE	317	17.674	11.509	77.532
2168	CG	PHE	317	16.927	10.791	76.402
2169	CD1	PHE	317	17.606	10.279	75.302
2170	CD2	PHE	317	15.550	10.642	76.488
2171	CE1	PHE	317	16.915	9.624	74.292
2172	CE2	PHE	317	14.857	9.982	75.480
2173	CZ	PHE	317	15.539	9.473	74.383
2174	N	VAL	318	17.456	14.039	79.418
2175	CA	VAL	318	17.640	14.825	80.648
2176	C	VAL	318	17.956	13.924	81.859
2177	O	VAL	318	18.158	12.723	81.678
2178	CB	VAL	318	16.307	15.558	80.759
2179	CG1	VAL	318	15.182	14.547	80.920
2180	CG2	VAL	318	16.228	16.693	81.779
2181	N	LEU	319	18.173	14.513	83.029
2182	CA	LEU	319	18.519	13.764	84.250
2183	C	LEU	319	17.325	13.121	84.950
2184	O	LEU	319	17.226	11.889	85.022
2185	CB	LEU	319	19.155	14.751	85.232
2186	CG	LEU	319	19.538	14.140	86.584
2187	CD1	LEU	319	20.371	12.874	86.435
2188	CD2	LEU	319	20.254	15.166	87.455
2189	N	GLY	320	16.411	13.951	85.423
2190	CA	GLY	320	15.371	13.460	86.333
2191	C	GLY	320	13.961	13.514	85.762
2192	O	GLY	320	13.016	13.013	86.388
2193	N	ARG	321	13.830	14.071	84.571
2194	CA	ARG	321	12.535	14.095	83.886
2195	C	ARG	321	12.339	12.772	83.150
2196	O	ARG	321	12.595	12.652	81.947
2197	CB	ARG	321	12.547	15.269	82.916
2198	CG	ARG	321	12.932	16.602	83.561
2199	CD	ARG	321	11.781	17.348	84.238
2200	NE	ARG	321	11.328	16.718	85.489
2201	CZ	ARG	321	10.072	16.796	85.934
2202	NH1	ARG	321	9.171	17.523	85.268
2203	NH2	ARG	321	9.726	16.174	87.063

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2204	N	ASP	322	11.940	11.770	83.914
2205	CA	ASP	322	11.892	10.399	83.414
2206	C	ASP	322	10.524	9.768	83.665
2207	O	ASP	322	9.543	10.163	83.020
2208	CB	ASP	322	13.003	9.633	84.138
2209	CG	ASP	322	13.435	8.322	83.471
2210	OD1	ASP	322	14.604	8.012	83.646
2211	OD2	ASP	322	12.571	7.533	83.136
2212	N	PHE	323	10.433	9.011	84.757
2213	CA	PHE	323	9.428	7.940	85.049
2214	C	PHE	323	9.079	6.962	83.895
2215	O	PHE	323	8.923	5.764	84.152
2216	CB	PHE	323	8.168	8.497	85.735
2217	CG	PHE	323	7.224	9.441	84.988
2218	CD1	PHE	323	7.310	10.815	85.180
2219	CD2	PHE	323	6.234	8.921	84.163
2220	CE1	PHE	323	6.439	11.665	84.513
2221	CE2	PHE	323	5.365	9.771	83.493
2222	CZ	PHE	323	5.470	11.144	83.666
2223	N	ARG	324	8.886	7.470	82.691
2224	CA	ARG	324	8.779	6.715	81.450
2225	C	ARG	324	9.759	7.430	80.499
2226	O	ARG	324	10.751	7.927	81.049
2227	CB	ARG	324	7.302	6.700	81.065
2228	CG	ARG	324	6.590	5.553	81.768
2229	CD	ARG	324	7.204	4.226	81.335
2230	NE	ARG	324	6.693	3.103	82.133
2231	CZ	ARG	324	7.194	1.868	82.053
2232	NH1	ARG	324	8.165	1.596	81.178
2233	NH2	ARG	324	6.696	0.896	82.820
2234	N	PRO	325	9.665	7.368	79.175
2235	CA	PRO	325	10.573	8.193	78.366
2236	C	PRO	325	10.425	9.699	78.597
2237	O	PRO	325	10.663	10.219	79.698
2238	CB	PRO	325	10.266	7.866	76.942
2239	CG	PRO	325	9.116	6.883	76.889
2240	CD	PRO	325	8.769	6.567	78.330
2241	N	TRP	326	10.319	10.382	77.467
2242	CA	TRP	326	9.917	11.796	77.360
2243	C	TRP	326	11.095	12.786	77.415
2244	O	TRP	326	11.124	13.684	78.268
2245	CB	TRP	326	8.831	12.154	78.372
2246	CG	TRP	326	7.735	11.122	78.634
2247	CD1	TRP	326	7.336	10.694	79.877
2248	CD2	TRP	326	6.942	10.378	77.678

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2249	NE1	TRP	326	6.380	9.754	79.731
2250	CE2	TRP	326	6.123	9.518	78.433
2251	CE3	TRP	326	6.890	10.353	76.290
2252	CZ2	TRP	326	5.272	8.632	77.787
2253	CZ3	TRP	326	6.033	9.463	75.655
2254	CH2	TRP	326	5.228	8.610	76.395
2255	N	LEU	327	12.114	12.420	76.643
2256	CA	LEU	327	13.174	13.252	75.991
2257	C	LEU	327	13.300	14.769	76.286
2258	O	LEU	327	12.377	15.400	76.811
2259	CB	LEU	327	12.716	13.079	74.540
2260	CG	LEU	327	13.584	13.628	73.425
2261	CD1	LEU	327	14.809	12.752	73.208
2262	CD2	LEU	327	12.760	13.728	72.151
2263	N	ILE	328	14.461	15.351	75.983
2264	CA	ILE	328	14.535	16.826	75.909
2265	C	ILE	328	14.959	17.365	74.525
2266	O	ILE	328	14.852	18.583	74.319
2267	CB	ILE	328	15.380	17.426	77.037
2268	CG1	ILE	328	16.744	16.768	77.218
2269	CG2	ILE	328	14.591	17.446	78.342
2270	CD1	ILE	328	17.809	17.384	76.321
2271	N	GLU	329	15.495	16.507	73.656
2272	CA	GLU	329	15.757	16.843	72.229
2273	C	GLU	329	16.406	15.683	71.456
2274	O	GLU	329	17.395	15.097	71.912
2275	CB	GLU	329	16.658	18.075	72.065
2276	CG	GLU	329	18.032	17.917	72.704
2277	CD	GLU	329	18.999	18.977	72.187
2278	OE1	GLU	329	18.534	19.970	71.649
2279	OE2	GLU	329	20.198	18.737	72.288
2280	N	ILE	330	15.850	15.340	70.304
2281	CA	ILE	330	16.503	14.354	69.427
2282	C	ILE	330	17.438	15.000	68.407
2283	O	ILE	330	17.009	15.540	67.380
2284	CB	ILE	330	15.442	13.557	68.672
2285	CG1	ILE	330	14.670	12.637	69.595
2286	CG2	ILE	330	16.047	12.737	67.542
2287	CD1	ILE	330	13.565	11.922	68.831
2288	N	ASN	331	18.726	14.899	68.675
2289	CA	ASN	331	19.709	15.292	67.672
2290	C	ASN	331	19.815	14.239	66.576
2291	O	ASN	331	19.484	13.057	66.757
2292	CB	ASN	331	21.053	15.547	68.337
2293	CG	ASN	331	20.930	16.806	69.188



Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2294	OD1	ASN	331	20.186	17.733	68.839
2295	ND2	ASN	331	21.655	16.827	70.291
2296	N	SER	332	20.156	14.748	65.406
2297	CA	SER	332	20.239	13.966	64.163
2298	C	SER	332	21.281	12.852	64.208
2299	O	SER	332	21.961	12.634	65.216
2300	CB	SER	332	20.588	14.924	63.030
2301	OG	SER	332	21.866	15.483	63.310
2302	N	SER	333	21.392	12.153	63.090
2303	CA	SER	333	22.332	11.023	62.981
2304	C	SER	333	23.841	11.342	63.121
2305	O	SER	333	24.512	10.468	63.684
2306	CB	SER	333	22.080	10.269	61.673
2307	OG	SER	333	22.403	11.096	60.561
2308	N	PRO	334	24.409	12.472	62.687
2309	CA	PRO	334	25.787	12.781	63.105
2310	C	PRO	334	25.899	13.269	64.552
2311	O	PRO	334	26.039	14.474	64.796
2312	CB	PRO	334	26.247	13.870	62.191
2313	CG	PRO	334	25.039	14.451	61.484
2314	CD	PRO	334	23.872	13.562	61.856
2315	N	THR	335	25.847	12.345	65.492
2316	CA	THR	335	26.129	12.675	66.892
2317	C	THR	335	27.637	12.610	67.123
2318	O	THR	335	28.315	11.808	66.479
2319	CB	THR	335	25.399	11.689	67.795
2320	OG1	THR	335	25.927	10.388	67.591
2321	CG2	THR	335	23.911	11.660	67.484
2322	N	MET	336	28.163	13.538	67.906
2323	CA	MET	336	29.613	13.569	68.183
2324	C	MET	336	30.016	12.792	69.429
2325	O	MET	336	29.833	13.260	70.559
2326	CB	MET	336	30.084	15.010	68.350
2327	CG	MET	336	30.195	15.773	67.033
2328	SD	MET	336	31.506	15.261	65.891
2329	CE	MET	336	30.663	13.980	64.934
2330	N	HIS	337	30.642	11.652	69.193
2331	CA	HIS	337	31.210	10.823	70.243
2332	C	HIS	337	32.481	10.123	69.722
2333	O	HIS	337	32.408	9.203	68.899
2334	CB	HIS	337	30.130	9.826	70.684
2335	CG	HIS	337	29.545	8.914	69.618
2336	ND1	HIS	337	29.928	7.653	69.341
2337	CD2	HIS	337	28.507	9.210	68.766
2338	CE1	HIS	337	29.178	7.169	68.331

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2339	NE2	HIS	337	28.303	8.135	67.973
2340	N	PRO	338	33.631	10.674	70.080
2341	CA	PRO	338	34.941	10.135	69.681
2342	C	PRO	338	35.190	8.690	70.107
2343	O	PRO	338	34.277	7.941	70.475
2344	CB	PRO	338	35.959	11.036	70.308
2345	CG	PRO	338	35.252	12.158	71.039
2346	CD	PRO	338	33.767	11.913	70.843
2347	N	SER	339	36.473	8.362	70.165
2348	CA	SER	339	36.975	6.984	70.362
2349	C	SER	339	36.921	6.455	71.802
2350	O	SER	339	37.359	5.328	72.078
2351	CB	SER	339	38.427	6.959	69.896
2352	OG	SER	339	38.468	7.357	68.530
2353	N	THR	340	36.221	7.168	72.661
2354	CA	THR	340	36.253	6.848	74.083
2355	C	THR	340	35.607	5.503	74.535
2356	O	THR	340	36.196	4.938	75.467
2357	CB	THR	340	35.628	8.024	74.820
2358	OG1	THR	340	36.060	9.234	74.207
2359	CG2	THR	340	36.071	8.054	76.272
2360	N	PRO	341	34.618	4.873	73.881
2361	CA	PRO	341	34.101	3.619	74.460
2362	C	PRO	341	35.125	2.483	74.489
2363	O	PRO	341	35.350	1.923	75.570
2364	CB	PRO	341	32.927	3.225	73.620
2365	CG	PRO	341	32.776	4.190	72.466
2366	CD	PRO	341	33.856	5.231	72.664
2367	N	VAL	342	35.936	2.389	73.445
2368	CA	VAL	342	36.922	1.310	73.329
2369	C	VAL	342	38.249	1.671	74.007
2370	O	VAL	342	39.095	0.803	74.244
2371	CB	VAL	342	37.151	1.074	71.839
2372	CG1	VAL	342	37.930	-0.211	71.581
2373	CG2	VAL	342	35.823	1.031	71.095
2374	N	THR	343	38.404	2.937	74.363
2375	CA	THR	343	39.615	3.368	75.069
2376	C	THR	343	39.413	3.427	76.582
2377	O	THR	343	40.337	3.819	77.305
2378	CB	THR	343	40.064	4.734	74.563
2379	OG1	THR	343	39.033	5.675	74.827
2380	CG2	THR	343	40.346	4.723	73.065
2381	N	ALA	344	38.215	3.116	77.049
2382	CA	ALA	344	37.975	3.074	78.493
2383	C	ALA	344	37.489	1.697	78.928

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2384	O	ALA	344	38.005	1.117	79.893
2385	CB	ALA	344	36.939	4.125	78.844
2386	N	GLN	345	36.506	1.183	78.208
2387	CA	GLN	345	36.038	-0.185	78.440
2388	C	GLN	345	36.031	-0.987	77.146
2389	O	GLN	345	36.742	-0.688	76.181
2390	CB	GLN	345	34.653	-0.233	79.081
2391	CG	GLN	345	34.682	-0.143	80.607
2392	CD	GLN	345	34.094	1.187	81.069
2393	OE1	GLN	345	34.427	2.234	80.506
2394	NE2	GLN	345	33.137	1.114	81.981
2395	N	LEU	346	35.168	-1.987	77.140
2396	CA	LEU	346	35.212	-3.044	76.123
2397	C	LEU	346	34.089	-3.014	75.080
2398	O	LEU	346	33.690	-4.089	74.618
2399	CB	LEU	346	35.151	-4.375	76.866
2400	CG	LEU	346	36.300	-4.523	77.862
2401	CD1	LEU	346	36.084	-5.721	78.780
2402	CD2	LEU	346	37.647	-4.616	77.152
2403	N	CYS	347	33.546	-1.859	74.733
2404	CA	CYS	347	32.458	-1.897	73.740
2405	C	CYS	347	32.633	-0.896	72.599
2406	O	CYS	347	33.133	0.222	72.776
2407	CB	CYS	347	31.111	-1.670	74.414
2408	SG	CYS	347	30.751	0.024	74.906
2409	N	ALA	348	32.171	-1.314	71.432
2410	CA	ALA	348	32.212	-0.460	70.242
2411	C	ALA	348	30.833	-0.331	69.598
2412	O	ALA	348	30.452	-1.160	68.760
2413	CB	ALA	348	33.191	-1.059	69.238
2414	N	GLN	349	30.240	0.841	69.763
2415	CA	GLN	349	28.895	1.084	69.222
2416	C	GLN	349	28.875	1.287	67.706
2417	O	GLN	349	27.894	0.889	67.067
2418	CB	GLN	349	28.285	2.285	69.937
2419	CG	GLN	349	29.212	3.493	69.966
2420	CD	GLN	349	28.675	4.535	70.943
2421	OE1	GLN	349	29.444	5.336	71.491
2422	NE2	GLN	349	27.378	4.476	71.190
2423	N	VAL	350	30.032	1.561	67.122
2424	CA	VAL	350	30.125	1.665	65.665
2425	C	VAL	350	30.313	0.289	65.011
2426	O	VAL	350	29.847	0.074	63.884
2427	CB	VAL	350	31.253	2.634	65.327
2428	CG1	VAL	350	32.529	2.327	66.102

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2429	CG2	VAL	350	31.506	2.700	63.828
2430	N	GLN	351	30.659	-0.697	65.824
2431	CA	GLN	351	30.724	-2.071	65.338
2432	C	GLN	351	29.356	-2.721	65.517
2433	O	GLN	351	28.942	-3.534	64.683
2434	CB	GLN	351	31.794	-2.813	66.124
2435	CG	GLN	351	31.962	-4.246	65.641
2436	CD	GLN	351	33.177	-4.856	66.327
2437	OE1	GLN	351	33.108	-5.294	67.481
2438	NE2	GLN	351	34.298	-4.803	65.629
2439	N	GLU	352	28.559	-2.118	66.385
2440	CA	GLU	352	27.157	-2.516	66.514
2441	C	GLU	352	26.315	-1.836	65.425
2442	O	GLU	352	25.337	-2.430	64.957
2443	CB	GLU	352	26.677	-2.163	67.919
2444	CG	GLU	352	25.332	-2.808	68.240
2445	CD	GLU	352	25.020	-2.664	69.728
2446	OE1	GLU	352	25.757	-3.237	70.519
2447	OE2	GLU	352	24.031	-2.019	70.052
2448	N	ASP	353	26.857	-0.776	64.837
2449	CA	ASP	353	26.287	-0.198	63.609
2450	C	ASP	353	26.582	-1.083	62.404
2451	O	ASP	353	25.725	-1.228	61.524
2452	CB	ASP	353	26.920	1.161	63.316
2453	CG	ASP	353	26.445	2.246	64.268
2454	OD1	ASP	353	25.317	2.682	64.103
2455	OD2	ASP	353	27.267	2.752	65.021
2456	N	THR	354	27.652	-1.856	62.498
2457	CA	THR	354	28.018	-2.786	61.429
2458	C	THR	354	27.180	-4.061	61.516
2459	O	THR	354	26.836	-4.647	60.482
2460	CB	THR	354	29.498	-3.124	61.579
2461	OG1	THR	354	30.230	-1.907	61.637
2462	CG2	THR	354	30.017	-3.950	60.409
2463	N	ILE	355	26.629	-4.313	62.693
2464	CA	ILE	355	25.691	-5.425	62.868
2465	C	ILE	355	24.300	-5.052	62.350
2466	O	ILE	355	23.596	-5.909	61.797
2467	CB	ILE	355	25.634	-5.757	64.356
2468	CG1	ILE	355	27.017	-6.143	64.865
2469	CG2	ILE	355	24.636	-6.875	64.635
2470	CD1	ILE	355	26.998	-6.447	66.358
2471	N	LYS	356	24.047	-3.756	62.249
2472	CA	LYS	356	22.788	-3.268	61.679
2473	C	LYS	356	22.812	-3.191	60.150

Atom No.	Atom Type	Residue	Residue Position	X Coord.	Y Coord.	Z Coord.
2474	O	LYS	356	21.759	-2.975	59.538
2475	CB	LYS	356	22.466	-1.898	62.253
2476	CG	LYS	356	22.311	-1.948	63.767
2477	CD	LYS	356	21.764	-0.623	64.272
2478	CE	LYS	356	22.616	0.538	63.776
2479	NZ	LYS	356	21.991	1.830	64.100
2480	N	VAL	357	23.923	-3.582	59.537
2481	CA	VAL	357	24.013	-3.651	58.069
2482	C	VAL	357	23.321	-4.910	57.513
2483	O	VAL	357	22.993	-4.963	56.319
2484	CB	VAL	357	25.493	-3.609	57.687
2485	CG1	VAL	357	25.719	-3.737	56.184
2486	CG2	VAL	357	26.135	-2.324	58.198
2487	N	ALA	358	22.844	-5.759	58.416
2488	CA	ALA	358	22.034	-6.927	58.043
2489	C	ALA	358	20.614	-6.565	57.581
2490	O	ALA	358	19.973	-7.382	56.910
2491	CB	ALA	358	21.950	-7.852	59.252
2492	N	VAL	359	20.236	-5.298	57.713
2493	CA	VAL	359	18.957	-4.804	57.182
2494	C	VAL	359	19.015	-4.598	55.656
2495	O	VAL	359	17.977	-4.491	54.996
2496	CB	VAL	359	18.652	-3.488	57.899
2497	CG1	VAL	359	17.348	-2.850	57.431
2498	CG2	VAL	359	18.612	-3.701	59.409
2499	N	ASP	360	20.206	-4.720	55.085
2500	CA	ASP	360	20.381	-4.668	53.627
2501	C	ASP	360	20.198	-6.020	52.929
2502	O	ASP	360	20.471	-6.117	51.725
2503	CB	ASP	360	21.769	-4.126	53.312
2504	CG	ASP	360	21.791	-2.621	53.541
2505	OD1	ASP	360	21.997	-2.212	54.676
2506	OD2	ASP	360	21.442	-1.914	52.606
2507	N	ARG	361	19.776	-7.044	53.658
2508	CA	ARG	361	19.529	-8.365	53.061
2509	C	ARG	361	18.211	-8.398	52.275
2510	O	ARG	361	17.150	-8.746	52.808
2511	CB	ARG	361	19.479	-9.373	54.203
2512	CG	ARG	361	19.349	-10.811	53.717
2513	CD	ARG	361	19.255	-11.771	54.899
2514	NE	ARG	361	20.449	-11.670	55.754
2515	CZ	ARG	361	20.405	-11.368	57.054
2516	NH1	ARG	361	19.235	-11.106	57.642
2517	NH2	ARG	361	21.536	-11.299	57.760